

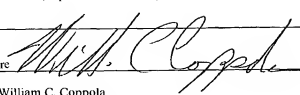
FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV. 5-93)		ATTORNEY'S DOCKET NUMBER  <b>ST99021 US PCT</b>  U.S. APPLICATION NO. <b>10/018729</b>
<b>TRANSMITTAL LETTER TO THE UNITED STATES          DESIGNATED/ELECTED OFFICE (DO/EO/US)          CONCERNING A FILING UNDER 35 U.S.C. 371</b>		
INTERNATIONAL APPLICATION NO.  PCT/FR00/01744	INTERNATIONAL FILING DATE  22 June 2000	PRIORITY DATE CLAIMED  France 99/07,957 (22.06.1999) US 60/149,721 (20.08.1999)
TITLE OF INVENTION  <b>SYSTEM FOR REGULATION OF EXPRESSION USING PPAR NUCLEAR RECEPTORS</b>		
APPLICANT(S) FOR DO/EO/US  DARTEIL, Raphael; CROUZET, Joel; STAELS, Bart; MAHFOUDI, Abderrahim		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US) 6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has <b>NOT</b> expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input checked="" type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)), and Power of Attorney (unexecuted). 10. <input checked="" type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).		
Items 11. to 16. Below concern other document(s) or information included: 11. <input type="checkbox"/> An information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input type="checkbox"/> A <b>FIRST</b> preliminary amendment. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input checked="" type="checkbox"/> Other items or information Print EFS		
<b>CERTIFICATION UNDER 37 CFR 1.10</b>		
"Express Mail" Mailing Number EV 047246531 US I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated Above and is addressed to Box PCT, Commissioner for Patents, Washington, DC 20231, Attn. EO/US William C. Coppola (Type or print name of person mailing paper)		Date of Deposit December 18, 2001  <i>Sally Kapadia</i> (Signature of person mailing paper)
U.S. APPLICATION NO. (If Known, see C.F.R. 1.5)	INTERNATIONAL APPLICATION NO. PCT/FR00/01744	ATTORNEY'S DOCKET NUMBER ST99021 US PCT

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17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO.....\$ 860.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) .....\$ 720.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))..\$ 790.00 Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid USPTO.....\$1070.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....\$ 98.00				CALCULATIONS		PTO use only	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$ 860.00			
Surcharge of \$130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(c)).				\$ 0.00			
Claims	Number Filed	Number Extra	Rate				
Total Claims	35 - 20 =	15	X \$ 18.00	\$ 270.00			
Independent Claims	- 3 =		X \$ 80.00	\$ 0.00			
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$ 270.00			
TOTAL OF ABOVE CALCULATIONS =				\$1130.00			
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$			
SUBTOTAL =				\$1130.00			
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VIA EXPRESS MAIL # E104724631 US

SYSTEM FOR REGULATION OF EXPRESSION USING PPAR NUCLEAR  
RECEPTORS

The present invention relates to the field of biology. It relates in particular to the field of the regulation of the expression of genes and, more particularly, it describes the design and development of a new system for the pharmacological regulation of the expression of transgenes. The invention is based in particular on the use of constructs of human origin for activating the transcription of the transgene. The invention thus describes new compositions, constructs and methods allowing the effective regulation of the expression of a nucleic acid in vitro, ex vivo or in vivo, for example in muscle cells. The applications which result from the present invention are many, in the experimental, clinical, therapeutic or diagnostic fields, for example.

Controlling the level and the duration of the expression of transgenes is necessary for many applications. Thus, in gene therapy, the success of the therapy may require a specific assay of the protein synthesized from the transgene. Likewise, the production of recombinant proteins in vitro may be improved using inducible expression systems, allowing for example uncoupling of the growth and production phases. The construction of transgenic animals, the study of the effects of a gene and of the

bioavailability of a protein, and the like, are so many situations in which an appropriate control of genetic expression may be used and may provide improvement.

Various artificial regulators of transcription have been designed in the prior art, which are activated by a xenobiotic molecule which bind to the promoter sequences for transcription of the transgene.

A first illustration of these regulators was constructed by fusion of the E. coli Lac repressor with the herpes simplex virus (HSV) VP16 transactivator domain. Two versions of these regulators exist, one capable of being activated by isopropyl  $\beta$ -D-thiogalactoside (IPTG) and the other inactivated by IPTG (Baim S. et al., *Proc Natl Acad Sci USA*, **88** (1991) 5072-5076; Labow M. et al., *Mol. Cell. Biol.*, **10** (1990) 3343-3356).

Another system was constructed by fusion of the E. coli Tet repressor with the HSV VP16 transactivator domain. Two versions of these regulators also exist, one capable of being activated by tetracycline or its derivatives and the other inactivated by these same molecules (Gossen M. and Bujard H., *Proc Natl Acad Sci USA*, **89** (1992) 5547-5551; Gossen M. et al., *Science*, **286** (1995) 1766-1769).

Another system was constructed by fusion of the DNA-binding domain of the S. cerevisiae GAL4

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protein with the ligand-binding domain of the human progesterone receptor and the HSV VP16 transactivator domain; this version is activated by a progesterone analogue such as RU486 (Wang Y. et al., *Proc Natl Acad Sci USA*, **91** (1994) 8180-8184). A fusion of the drosophila ecdysone receptor with the HSV VP16 transactivator domain has also been described, activated by ecdysone and the analogues of this steroid hormone (No D et al., *Proc Natl Acad Sci USA*, **93** (1996) 3346-3351). Another system takes advantage of the capacity of certain immunosuppressive molecules (cyclosporin A, rapamycin and its derivatives) to promote the combination of certain cellular proteins. A transcriptional regulator then consists of two protein subunits; the first may be formed by the fusion of a chimeric DNA-binding domain and of three copies of the human FKBP protein and the second by the fusion of the rapamycin-binding domain of the human FRAP protein and of the transactivator domain of the human NFkB p65 subunit. This transcriptional regulator is activated by rapamycin which allows the dimerization of the two subunits (Rivera V. et al., *Nat. Med.*, **2** (1996) 1028-1032).

Although these systems make it possible to obtain satisfactory levels of regulation in some tissues, they exhibit, nevertheless, certain disadvantages which limit their conditions of use.

Thus, these transcriptional regulators are xenogenic proteins in humans. They indeed consist of protein fragments obtained from bacteria, viruses, yeasts or insects or, when the protein domains are of human origin, their joining creates sequences which are foreign to humans. These protein domains may therefore induce a cytotoxic immune reaction, causing the destruction of the cells which express the gene of interest under the control of the xenogenic transcriptional regulator, and thus the termination of the expression of the transgene. This situation may necessitate the use of repeated administrations of the therapeutic gene, which constitutes a major disadvantage, in particular when this involves a traumatic surgical act, and which is not always effective, in particular when the vector of the therapeutic gene is a virus whose first injection causes an immune reaction. In addition, the levels of expression observed with the prior art systems of regulation are not always satisfactory.

The need therefore exists for an improved system of regulating expression, compatible with use in vivo, which can be used in various tissues, and which ensures high levels of expression in the activated state. The present invention provides a solution to these problems.

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The present invention indeed relates to a system of regulation using an activator of human origin. This should make it possible to avoid the repeated administrations of the therapeutic gene.

5           The present invention describes in particular an improved system of inducible expression using the PPAR (Peroxisome proliferator-activated receptors) nuclear receptors as transcriptional regulators. The use of PPRE in a hepatospecific expression system has  
10 been described in application WO 98/21349. The improved system according to the invention currently makes it possible to produce the transcriptional regulator (a PPAR protein of human origin, and therefore essentially nonxenogenic in humans) and the inducible promoter,  
15 which controls the expression of the transgene, is composed, on the one hand, of a minimum promoter and, on the other hand, of a PPAR response element (PPRE). The system of the invention is activable, in vitro and also in vivo, in particular in the muscle, by the  
20 ligands specific for the PPARs. Furthermore, the level of expression of the transgene, obtained after activation, is comparable to that of a strong promoter such as the hCMV-IE promoter.

          The system according to the present invention  
25 therefore exhibits numerous advantages, simultaneously in terms of substantial induction, of tolerance (in

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particular for an in vivo use), of strength and of conditions of use.

The invention therefore describes new constructs for the preparation and the use of this system, in particular promoter regions, expression cassettes and plasmids. The invention also describes new PPAR constructs allowing an improved control of the expression of genes, as well as combinations of these different constructs. The invention shows, in addition, that these methods and compositions allow substantial control and regulation of the expression in vitro and in vivo. The invention also relates to cells comprising constructs of the invention, as well as methods for screening compounds which are ligands for PPARs, for example.

More particularly, a first subject of the invention consists in a composition comprising:

(a) a first element comprising a nucleic acid of interest under the control of an inducible promoter comprising a PPAR response element and a minimal transcriptional promoter, and

(b) a second element comprising a nucleic acid encoding a PPAR under the control of a transcriptional promoter, for their use simultaneously, separately or spaced out over time.



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In a more specific mode, the compositions of the invention comprise in addition:

(c) a ligand for PPAR,  
also for a use simultaneously, separately or spaced out  
5 over time.

Advantageously, they comprise, in addition, an element (d) comprising a nucleic acid encoding a retinoid X receptor (RXR) under the control of a transcriptional promoter.

10 The expression for a use simultaneously, separately or spaced out over time indicates that the elements (a), (b) and, where appropriate, (c) and/or (d) may be prepared separately, packaged separately, and used sequentially, to allow control of the  
15 expression of the nucleic acid of interest. Typically, the elements (a) and (b), and optionally (d) are prepared and packaged together, whereas the compound (c) is packaged separately and used spaced out over time with (a) and (b), and optionally (d), the  
20 combination of these different elements in a cell, a tissue, an organ and the like leading to the desired effect of regulation of expression.

In this regard, in a specific embodiment of a composition of the invention, the elements (a), (b) and  
25 optionally (d) are carried by distinct genetic constructs.

In another specific and preferred embodiment of a composition of the invention, the elements (a), (b) and optionally (d) are assembled in the same genetic construct. The present invention thus describes  
5 complex genetic constructs allowing the expression of a product of interest and of a PPAR. These constructs are particularly advantageous since they contain, on their own, all the genetic elements necessary for the regulated expression of the nucleic acid of interest.

10 The genetic construct(s) may be of a varied, in particular plasmid, episomal, chromosomal, viral or phage, nature and/or origin, and the like. Preferably, the genetic construct is a plasmid or viral vector.

By way of illustration of plasmids separately  
15 carrying the elements (a) or (b), there may be mentioned for example the plasmids JxnS-TK-pGL3, JxnAS-TK-pGL3, DR1xnS-TK-pGL3, DR1xnAS-TK-pGL3, JxnAS-CMV-pGL3, pSG5-hPPARG2g2 or Jx10AS-CMV-EF-pGL3, which will be described in detail later.

20 By way of illustration of plasmids in which the elements (a) and (b) have been assembled, there may be mentioned for example the plasmids Jx5AS-TK-Luc-hPPARG2, SV-g2-J10-C-pGL3, hPPARG2-CMV-Jx5AS-TK-pGL3 or hPPARG2-CMV-Jx10AS-CMV-pGL3, which will be described in  
25 detail later.

As an example of a viral vector, there may be mentioned in particular a recombinant adenovirus, a

recombinant retrovirus, an AAV, a herpesvirus, a vaccinia virus and the like, whose preparation may be carried out according to methods known to persons skilled in the art.

5           The arrangement and the structure of the genetic constructs will be described in greater detail in the text which follows.

          In this regard, as indicated above, the element (a) comprises an inducible promoter comprising  
10 at least:

- a PPAR response element, and
- a minimal transcriptional promoter.

          A PPAR response element (PPRE, "Peroxisome Proliferator Response Element") is a nucleic acid  
15 region capable of binding a PPAR, it being possible for the binding of the PPAR to then mediate a signal around neighbouring nucleic regions. A PPAR response element is therefore a nucleic acid region capable of binding PPARs. For carrying out the invention, the PPAR  
20 response element comprises more particularly one or more PPAR-binding sites. Such binding sites have been described in the prior art, such as for example, in different human promoters (gene for apolipoprotein AII, for example). Such sites may also be artificially  
25 constructed, and tested for their PPRE properties, as is described below.

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In a specific embodiment, the PPAR response element comprises one or more sites having the sequence TCAACCTTTACCCTGGTAG (SEQ ID NO:1) or functional variants of this sequence. The sequence SEQ ID NO:1 corresponds to the J region of the human apoAII promoter (nucleotides -734 to -716).

In another specific embodiment, the PPAR response element comprises one or more sites having the sequence AGGTCAAAGGTCA (SEQ ID NO:5) or functional variants of this sequence. The sequence SEQ ID NO:5 corresponds to the consensus region DR1.

The term functional variant designates any modified sequence conserving the properties of PPRE as mentioned above, that is to say in particular the capacity to bind a PPAR. The modifications may comprise one or more additions, mutations, deletions and/or substitutions of nucleotides in the sequence considered. These modifications may be introduced by conventional molecular biology methods, such as in particular site-directed mutagenesis or, more practically, by artificial synthesis of the sequence in a synthesizer. Generally, the variants conserve at least 50% of the residues of the initial sequence indicated. More preferably, the variants possess modifications affecting less than 5 nucleotides in the sequence considered. The variants thus obtained are

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then tested for their PPRE activity. This property may be checked in various ways, and in particular:

- (i) by bringing the test sequence into contact with a PPAR and a retinoid X receptor (RXR),  
5 preferably in an acellular test, and detecting the formation of a complex (for example by gel migration retardation);
- (ii) by inserting the test sequence into an expression cassette comprising a minimal promoter and a  
10 reporter gene, introducing the cassette into a cell, and detecting (where appropriate assaying) the expression of the reporter gene in the presence and in the absence of a PPAR and of a ligand for a PPAR;
- (iii) by any other technique known to a  
15 person skilled in the art, making it possible to detect the interaction between a nucleic acid and a protein, for example.

A variant is considered to be functional for the purpose of the present invention when the activity  
20 measured, for example in (ii) above, is preferably at least equal to 50% of that measured with a site having the sequence SEQ ID NO:1 or 5, more preferably at least equal to 75%. Functional variants of PPAR-binding sites for the purpose of the invention are described, for  
25 example, in Juge-Aubry et al., (J. Biol. Chem. 272 (1997) 25252) and in Nakshatri et al. (NAR 26 (1998)

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2491), which are incorporated into the present application by way of reference.

The retinoid X receptors (RXR) are encoded by three genes RXR $\alpha$ , RXR $\beta$  and RXR $\gamma$ , whose isolation and  
5 sequence have been described (Mangelsdorf DJ et al. (1990), *Nature* **345**, 224-229; Mangelsdorf DJ et al. (1992), *Genes Dev* **6**, 329-344). Preferably, the element (d) encodes the human RXR $\alpha$ .

As regards the PPAR/RXR heterodimerization,  
10 two reviews may be consulted: Mangelsdorf DJ and Evans RM (1995), *Cell* **83**, 841-850 and Wilson TM and Wahli W (1997), *Current Opinion in Chemical Biology* **1**, 235-241. The article by Schulman IG et al. (1989), *Molecular and Cellular Biology* **18**, 3483-3494 describes  
15 transactivation by the PPAR $\gamma$ /RXR $\alpha$  heterodimer.

The use of the element (d) is capable of synergizing the activity of the element (b).

As indicated above, in the compositions according to the invention, the PPAR response element  
20 may comprise several sites for binding to a PPAR. This may be a repetition of the same site, or combinations of different sites, the repetition of identical sites being preferred. More particularly, the response element comprises up to 30 binding sites, preferably  
25 from 3 to 20, more preferably from 5 to 15. A preferred embodiment of the invention is a construct comprising from 10 to 15 binding sites, the results presented in

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the examples indeed show the advantageous properties of such constructs in terms of induction and of level of expression, in particular in the muscle cells.

For the preparation of an inducible promoter

5 according to element (a) of the compositions of the invention, the PPAR response element is combined with a transcriptional minimal promoter. The minimal promoter is a transcriptional promoter having a basal activity which is low or inexistant, and capable of being

10 increased in the presence of a transcriptional activator (interaction of a PPAR activated with the PPRE element). A minimal promoter may therefore be a promoter which is naturally weak in mammalian cells, that is to say which produces a nontoxic and/or an

15 insufficient expression in order to obtain a pronounced biological effect. Advantageously, a minimal promoter is a construct prepared from a native promoter, by deletion of a region or regions not essential for the transcriptional activity. Thus, this is preferably a

20 promoter comprising essentially a TATA box, generally of less than 160 nucleotides in size, centred around the codon for initiation of transcription. A minimal promoter can thus be prepared from strong or weak viral or cellular promoters such as for example the promoter

25 of the herpesvirus thymidine kinase (TK) gene, the CMV immediate promoter, the PGK promoter, the promoter of the muscle creatine kinase (MCK) gene, the promoters of

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the genes for the various skeletal muscle actin isoforms, the promoter of the desmin gene, the promoter of the vimentin gene, the promoters of the myosin light chain or heavy chain genes, and the like. Specific

5 examples of minimum promoters are represented by nucleotides -54 to +48 of the CMV or -105 to +56 of the TK promoter, for example. It is understood that any variant of these promoters or similar constructs from other promoters may be constructed by persons skilled

10 in the art and used within the framework of the present invention.

The minimal promoter (Pmin), the PPAR response element (PPRE) and the nucleic acid of interest (NA) are arranged functionally in the element

15 (a), that is to say such that the minimal promoter controls the expression of the nucleic acid of interest and that its activity is regulated by the PPRE element. Generally, these regions are therefore arranged in the following order, in the 5'→3' orientation: PPRE-Pmin-

20 NA. However, any other functional arrangement can be envisaged by persons skilled in the art without departing from the present invention.

In addition, the various functional domains above may be directly linked to each other, or

25 separated by nucleotides which do not significantly affect the regulated character of the promoter of the element (a). Such nucleotides may be neutral residues



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from the functional point of view, resulting, for example, from cloning steps (PCR ends, restriction sites, and the like). These nucleotides may also possess biological properties which make it possible to confer improved characteristics or performances to the system of the invention (enhancer of housekeeping genes, tissue specific enhancer, silencer, intron, splicing site, and the like). In this regard, in a specific embodiment of the invention, the inducible promoter comprises, in addition, an enhancer region. Such a region advantageously makes it possible to increase the levels of expression of the nucleic acid of interest. Such an enhancer (E) region is preferably positioned in 3' of the minimal promoter, between the latter and the nucleic acid of interest, according to the following scheme (5'→3'): PPRE-Pmin-E-NA.

Moreover, in the constructs of the invention, the minimal promoter and the PPAR response element may be present either in the same orientation (that is to say in the direction of transcription), or in the opposite orientation (that is to say that the PPAR response element is in the antisense orientation relative to transcription by the Pmin promoter). As illustrated in the examples, these two embodiments allow an effective control of the regulation of the expression in vitro and in vivo.

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As indicated above, the element (b) of the compositions according to the invention comprises at least:

- a nucleic acid encoding a PPAR,
- 5       - under the control of a second transcriptional promoter.

The PPARs belong to the superfamily of nuclear hormone receptors, and are grouped into three distinct groups, PPAR $\alpha$ , PPAR $\delta$  (also called NUC-1 or 10 PPAR $\beta$ ) and PPAR $\gamma$ . The isolation and the sequence of many human PPARs have been described in the literature (see in particular Sher T. et al., *Biochemistry*, **32** (1993) 5598-5604; Mukherjee R. et al., *J. Steroid Biochem. Molec. Biol.*, **51** (1994) 157-166; Fajas L. et al., *J. Biol. Chem.*, **272** (1997) 18779-18789; Mukherjee R. et al., *J. Biol. Chem.*, **272** (1997) 8071-8076; Schmidt A. et al., *Mol. Endocrinol.* **6** (1992) 1634-1641). The PPAR $\gamma$  promoter has, in addition, been recently cloned, as described by application WO 99/05161.

20       In a preferred embodiment of the invention, the nucleic acid encoding a PPAR encodes a human PPAR, in particular a PPAR $\alpha$  or a PPAR $\gamma$ . The results presented in the examples indeed show that the use of these molecules ensures for the system of the invention high 25 levels of regulation and expression, in particular in the muscle cells.

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According to a first embodiment, this is a PPAR $\alpha$  or a PPAR $\gamma$  in its native form, that is to say without modification of primary structure relative to the natural molecule.

5 According to another embodiment, this is a modified PPAR comprising several ligand-binding sites.

In this regard, the present invention describes and also has as subject any modified PPAR comprising several ligand-binding sites. More particularly, this is a PPAR $\alpha$  or a PPAR $\gamma$ , still more preferably a PPAR $\gamma$ . Preferably, the modified PPARs according to the invention comprise from 2 to 5 ligand-binding sites, more preferably from 2 to 4 binding sites. This is more particularly PPAR containing 2 to 5  
10 copies of the E and F domains involved in the binding to the ligand. The PPAR proteins contain different domains: the N-terminal A/B domain which contains a transactivating region not dependent on the ligand, the C domain which is the DNA-binding domain (DBD) and the  
15 D domain which is a hinge region, and the E/F domains which contain a transactivating region dependent on the ligand. The E/F domains are also called ligand-binding domain (LBD) (see in particular Schoonjans K. et al., *Biochim. Biophys. Acta*, **1302** (1996) 93-109). The limits  
20 of the E/F domains vary from one PPAR to another. By way of example, for the human PPAR $\gamma$ 2 isoform used, the E/F domain extends from amino acid 284 to amino acid

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505. The present invention now shows that it is possible to construct modified PPARs comprising several repeated E and F domains, and that these modified PPARs are functional and possess improved properties of

5 inducibility by the ligands for the PPARs. Such constructs therefore represent an embodiment and a specific subject of the present invention.

A typical example of modified PPAR according to the invention is a PPAR $\gamma$  containing 2 ligand-binding

10 sites (that is to say two E and F domains). The complete protein sequence of PPAR $\gamma$ 2 $\gamma$ 2 is represented on the sequence SEQ ID NO:24.

SEQ ID NO:24

MGETLGDSPIDPESDFTDTLSANISQEMTMVDTMPFWPTNFGISSVDLSVMEDHSHSFEDI  
 KPFTTVDFSSISTPHYEDIPFTRTDPVVDYKYDLKLQEQSAIKVEPASPPYYSEKTQLYN  
 KPHEEPSNSLMAIECRVCGDKASGPHYGVHACEGCKGFFRRTIRLKLIIYDRCDLNCRIHKK  
 RNKCOYCRFOKCLAVGMSHNAIRFGRMPOAEKEKLLAEISSDIDQLNPESADLRALAKHLYD  
 SYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIF  
 QGCQFRSVEAVQETITEYAKSTPGFVNLDLNDQVTLKYGVHEIIYHTMLASLMNKGVLISEG  
 QGFMTRFPLKSLRKPFPGDFMEPKFEFAVKFNALDLDSDLAIFIAVILSGDRPGLLNKVPRI  
 EDIQDNLQALQLKLNHPSSQLFAKLLQKMTDLRQIVTEHVQLLQVKKTTETDMSLHPL  
 LQEIYKLDLYAWAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIFQGC  
 QFRSVEAVQETITEYAKSTPGFVNLDLNDQVTLKYGVHEIIYHTMLASLMNKGVLISEGQGF  
 MTRFPLKSLRKPFPGDFMEPKFEFAVKFNALDLDSDLAIFIAVILSGDRPGLLNKVPRI  
 QDNLQALQLKLNHPSSQLFAKLLQKMTDLRQIVTEHVQLLQVKKTTETDMSLHPLLQ  
 15 IYKDLY

The invention also relates to any variant of the sequence SEQ ID NO:24 conserving a PPAR-type activity (the capacity to activate, in the presence of a PPAR ligand such as BRL49653, a promoter containing a PP

20 sequence). The variants are understood to mean any mutant, deletant and/or polypeptide containing one or more additional residues. Preferably a variant

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conserving at least 80% of the residues of the sequence  
ID NO:24.

In addition, the invention also relates to  
any nucleic acid encoding such a modified PPAR. This  
5 may be a DNA (in particular a cDNA or a synthetic or  
semisynthetic DNA) or an RNA. This DNA may be  
constructed according to conventional molecular biology  
methods known to persons skilled in the art (synthesis,  
ligations, screening of libraries and the like). It is  
10 advantageously any nucleic acid comprising a sequence  
encoding a polypeptide having the sequence SEQ ID  
NO:24, or hybridizing with a sequence encoding a  
polypeptide of SEQ ID NO:24, and encoding a polypeptide  
with a PPAR-type activity. In addition, this DNA may  
15 comprise a transcriptional promoter and/or terminator,  
for example.

The second transcriptional promoter,  
controlling the expression of the nucleic acid encoding  
the PPAR, may be any strong or weak, ubiquitous or  
20 selective, constitutive or regulated promoter which is  
functional in mammalian cells, in particular in human  
cells. This may be a domestic cellular promoter (i.e.,  
a mammalian, in particular a human, gene), a natural or  
synthetic, simple or complex, viral, bacterial, insect  
25 or plant promoter, and the like. Examples of  
appropriate promoters for this element (b) are in  
particular viral promoters (SV40 virus immediate

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promoter, CMV virus immediate promoter, retrovirus LTR, herpesvirus TK promoter) or cellular promoters (PGK, albumin or EFl $\alpha$  promoter, or promoter of genes which are highly expressed in the muscle such as: promoter of the muscle creatine kinase (MCK) gene, promoters of the genes of the various skeletal muscle actin isoforms, promoter of the desmin gene, promoter of the vimentin gene, promoters of the myosin light chain or heavy chain genes). Moreover, the promoter may be modified by introduction of one or more enhancer regions, such as the enhancer region of intron 2 of the beta-globin gene, enhancer of the CMV virus very early gene, EFl $\alpha$  enhancer, silencer region(s), regions conferring a tissue specificity (for example regions isolated from tissue-specific promoters such as: promoter of the muscle creatine kinase (MCK) gene, promoters of the genes of the various skeletal muscle actin isoforms, promoter of the desmin gene, promoter of the vimentin gene, promoters of the myosin light chain or heavy chain genes) or a regulable character, or by deletion of regions not essential to the activity, for example. Such promoters may be used to express the RXR, contained in the element (d).

Preferred examples of a second promoter are the viral promoters, in particular the SV40 virus early promoter and the CMV immediate promoter, or derivatives thereof.

Moreover, in a specific embodiment, when the elements (a) and (b), and optionally (d), are assembled in the same genetic construct, the second transcriptional promoter (of the element (b)) and the inducible promoter of the element (a), and optionally the promoter of the element (d), may be grouped so as to form only one common, in particular bidirectional, promoter region, as will be explained in detail in the text which follows.

To this effect, another subject of the present invention consists in a vector comprising an element (a) and an element (b), and optionally an element (d), as defined above.

According to a first variant of the invention, the elements (a) and (b), and optionally (d), are in the same orientation in the vector. Such a variant is illustrated for example by the plasmid SV-g2-J10-C-pGL3 (Figure 17).

According to another variant of the invention, the elements (a) and (b), and optionally (d), are in the opposite orientation in the vector. Such a variant is illustrated for example by the plasmids represented in Figures 16, 18 and 19. More preferably, in this variant embodiment, the inducible promoter of the element (a) and the transcriptional promoter of the element (b) are assembled in the vector to form a regulable bidirectional promoter. Such an

embodiment is illustrated for example by the plasmids represented in Figures 18 and 19.

In this regard, a specific subject of the invention consists in a vector characterized in that it comprises, in the 5'→3' direction, a first nucleic acid encoding a PPAR, a first minimal transcriptional promoter controlling the expression of the said first nucleic acid, one or more PPAR response element(s), a second minimal transcriptional promoter and, under the control of the said second minimal transcriptional promoter, a second nucleic acid encoding a product of interest.

This type of construct is advantageous since it allows the co-expression of the two nucleic acids in the same plasmid, and the amplification of this expression by the regulation of the two nucleic acids by the PPARs and their ligands.

The expression of the nucleic acid of interest in the compositions of the invention is generally activated in the presence of a PPAR ligand (element (c)). In this regard, according to the PPAR used, various types of ligands, natural or synthetic, may be used.

Thus, the PPAR $\alpha$ -activating ligands are for example the fibrates such as fibric acid and its analogues. As analogues of fibric acid, there may be mentioned in particular gemfibrozyl (Atherosclerosis



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114(1) (1995) 61), bezafibrate (Hepatology 21 (1995) 1025), ciprofibrate (BCE&M 9(4) (1995) 825), clofibrate (Drug Safety 11 (1994) 301), fenofibrate (Fenofibrate Monograph, Oxford Clinical Communications, 1995),  
5 clinofibrate (Kidney International. 44(6) (1993) 1352), pirinixic acid (Wy-14,643) or 5,8,11,14-eicosatetraenoic acid (ETYA). These various compounds are compatible with a biological and/or pharmacological use in vitro or in vivo.

10 The PPAR $\gamma$ -activating ligands may be chosen from natural and synthetic ligands. As natural ligands, there may be mentioned fatty acids and eicosanoids (for example linoleic acid, linolenic acid, 9-HODE, 5-HODE) and as synthetic ligands, there may be mentioned  
15 thiazolidinediones, such as in particular rosiglitazone (BRL49653), pioglitazone or troglitazone (see for example Krey G. et al., *Mol. Endocrinol.*, **11** (1997) 779-791 or Kliewer S. and Willson T., *Curr. Opin. in Gen. Dev.* **8** (1998) 576-581) or the compound RG12525.

20 Moreover, the compositions according to the invention may contain several PPAR activators in combination, and in particular a fibrate or a fibrate analogue combined with a retinoid.

The subject of the invention is also the use  
25 of a composition or of a vector as defined above for expressing a nucleic acid of interest in a cell ex vivo or in vitro.

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In this regard, the nucleic acid may be any nucleic acid (DNA, RNA) encoding a product of interest (RNA, protein, polypeptide, peptide and the like). It may be a product of interest in the food, therapeutic  
5 or vaccine sector, a marker, and the like.

The invention also relates to the use of a composition or of a vector as defined above for the preparation of a product intended for expressing a nucleic acid of interest in a cell in vivo.

10 The subject of the invention is also a method for the regulated expression of a nucleic acid in a cell, comprising bringing the said cell into contact with a composition or a vector as defined above.

For a use in vitro or ex vivo, the cells may  
15 be brought into contact with the compositions or vectors of the invention according to various protocols. Thus, the cells in culture may be incubated directly with the elements (a), (b) and (c), and optionally (d), of the invention, for example with a  
20 vector containing the elements (a) and (b) and in the presence of the ligand (c). Alternatively, the cells may be incubated in a first instance with the elements (a) and (b) and optionally (d) (in particular assembled in the same vector) and then, in a second instance  
25 (after culture and optionally selection of the modified cells), the element (c) may be added. This latter type of protocol makes it possible, for example, to uncouple

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the culture phase (or the cell expansion phase) from the nucleic acid expression phase. These experiments may be carried out in any appropriate medium and device, preferably in a plate, dish, flask, in the  
5 sterile condition. The quantities of cells, vector and ligand can be easily adapted by persons skilled in the art, on the basis of the information provided in the examples and of their general knowledge.

For a use in vivo, the cells (or organs,  
10 tissue, and the like) are brought into contact, by administration of the elements (a), (b) and (c), and optionally (d), in vivo, simultaneously, separately or spaced out over time. To this effect, the elements (a) and (b), and optionally (d), optionally in the form of  
15 a single genetic construct, are generally administered by the parenteral, in particular intramuscular, intravenous, subcutaneous, intradermal, intratumoral or stereotaxic route. The choice of the mode of administration may be guided by the application  
20 envisaged, the tissue targeted and/or the type of product of interest encoded by the transgene. For this administration, the compositions of the invention may comprise any agent promoting cellular transfection (cationic polymer, lipid and the like). In a specific  
25 mode, the compositions are administered by the intramuscular route, and the genetic constructs are

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used in the form of a "naked" nucleic acid, that is to say without added transfection agent.

Likewise, when the elements (a) and (b), and optionally (d), are introduced by means of viral  
5 vectors, no additional transfection agent is necessary.

As illustrated in the examples, the ligand (c) may be administered before, simultaneously or after the elements (a) and (b), and optionally (d).

In this regard, the administration of the  
10 ligand may be carried out by the oral, anal, intravenous, intraperitoneal or intramuscular route, for example.

The doses used may be adapted by persons skilled in the art, on the basis of the in vivo data  
15 published in the literature. Thus for example, for a form not soluble in water, typical doses of ligand such as BRL49653 are between 5 and 50 mg/kg, for example 30 mg/kg, which make it possible to obtain a plasma concentration close to about 15 µg/ml at least. For a  
20 water-soluble form of ligand, whose bioavailability is greater (for example a maleate salt of BRL49653), the typical doses are lower, generally less than 5 mg/kg, for example from 0.01 to 1 mg/kg. These doses can be quite obviously adapted by persons skilled in the art  
25 as a function of the constructs used, the ligands used, and the desired applications and effects. In general, the results presented in the examples advantageously

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show that the compositions of the invention make it possible to obtain in vivo a high and regulated expression, at ligand doses less than those normally used. In addition, although repeated administrations of  
5 ligand may be carried out, the results presented also show that the expression is high after a single dose of ligand.

Generally, the vector doses used may vary between 0.01 and 1000 µg, or more, depending on the  
10 desired applications.

The invention may be used for expressing a gene in various types of cells, tissues or organs, in vitro, ex vivo or in vivo. In particular, this may be a mammalian, preferably a human, cell, tissue or organ.  
15 By way of illustration, there may be mentioned muscle cells (or a muscle), hepatic cells (or the liver), cardiac cells (or the heart, the arterial or vascular wall), nerve cells (or the brain, the marrow and the like) or tumour cells (or a tumour).

20 Preferably, the constructs, compositions and method of the invention are used for the regulated expression of a nucleic acid in a muscle cell (or a muscle) in vitro, ex vivo or in vivo. The results presented in the examples illustrate more particularly  
25 the advantages of the invention in vivo or in vitro in this type of cells.

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The invention also relates to any cell modified by bringing into contact with a composition or a vector as defined above.

The invention also relates to the use of a  
5 composition, of a vector or of a cell as defined above, in which the nucleic acid of interest is a reporter gene (such as for example secreted alkaline phosphatase or luciferase) for the screening in vitro, ex vivo or in vivo (in particular in the muscle cells or a muscle)  
10 for PPAR ligands. In this regard, the invention also describes a method for identifying PPAR ligands comprising the bringing into contact of a cell as defined above with a test molecule (or composition), and the detection of the expression of the nucleic acid  
15 of interest (the latter being preferably a reporter gene). The expression may, in addition, be compared with that observed in the absence of test compound or in the presence of a reference ligand, in order to evaluate the activity of the compound tested.

20 The invention also relates to the use of a composition or of a vector as defined above, for the construction of transgenic animals, in particular of nonhuman mammals, useful for preclinical studies, or for studies of bioavailability, or labelling, and the  
25 like.

The present invention will be described in greater detail with the aid of the examples which

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follow and which should be considered as illustrative and nonlimiting.

#### LEGEND TO THE FIGURES

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**Figure 1 :** Schematic representation of the plasmid FTKpGL3.

**Figure 2 :** Schematic representation of the plasmid Jx3S-TK-pGL3.

**Figure 3 :** Schematic representation of the plasmid Jx3AS-TK-pGL3.

**Figure 4 :** Schematic representation of the plasmid DR1x3S-TK-pGL3.

**Figure 5 :** Schematic representation of the plasmid DR1x3AS-TK-pGL3.

20

**Figure 6 :** Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with :

(i) 10 ng of plasmid FTKpGL3 (**a**), or Jx3S-TK-pGL3 (**b**), or Jx3AS-TK-pGL3 (**c**), or DR1x3S-TK-pGL3 (**d**), or DR1x3AS-TK-pGL3 (**e**), (ii) increasing quantities of plasmid pSG5-hPPARG2, and (iii) 20 ng of plasmid pRL-

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null. The activity of each inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

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**Figure 7** : Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with :

- (i) 10 ng of plasmid FTKpGL3 (a), or Jx3S-TK-pGL3 (b),  
10 or Jx3AS-TK-pGL3 (c), or DR1x3S-TK-pGL3 (d), or  
DR1x3AS-TK-pGL3 (e), (ii) increasing quantities of  
plasmid pSG5-hPPAR $\alpha$ (Koz), and (iii) 20 ng of plasmid  
pRL-null. The activity of each inducible promoter  
represents the luciferase activity of Photinus pyralis  
15 normalized using the activity of Renilla reniformis  
luciferase.

**Figure 8** : Schematic representation of the plasmid  
Jx5AS-CMV-pGL3.

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**Figure 9** : Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with :

(i) 10 ng of plasmid Jx5AS-TK-pGL3 (a), or Jx5AS-CMV-  
25 pGL3 (b), (ii) increasing quantities of plasmid pSG5-  
hPPAR $\gamma$ 2, and (iii) 20 ng of plasmid pRL-null. The  
activity of each inducible promoter represents the



luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

**Figure 10** : Activities of the inducible promoters

5 evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with :

(i) 10 ng of plasmid JxnAS-TK-pGL3, (ii) 10 ng of plasmid pSG5-hPPARG2, and (iii) 20 ng of plasmid pRL-null. The activity of each inducible promoter

10 represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

**Figure 11** : Activities of the inducible promoters

15 evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with :

(i) 10 ng of plasmid JxnAS-CMV-pGL3, (ii) 10 ng (a) or 50 ng (b) of plasmid pSG5-hPPARG2, and (iii) 20 ng of plasmid pRL-null. The activity of each inducible

20 promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

**Figure 12** : Schematic representation of the plasmid

25 pSG5-hPPARG2g2.

**Figure 13** : Comparison of the transcriptional regulators hPPARG2 and hPPARG2g2. Mouse myoblasts (C2C12) are contransected with: (i) 10 ng of plasmid Jx10AS-CMV-pGL3, (ii) increasing quantities of plasmid pSG5-hPPARG2 (**a**) or pSG5-hPPARG2g2 (**b**), and (iii) 20 ng of plasmid pRL-null. The activity of the inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase. (**c**) : factors for induction by BRL49653 obtained with the plasmid pSG5-hPPARG2 or the plasmid pSG5-hPPARG2g2. This induction factor is calculated by dividing the activity in the presence of BRL49653 by the activity in the presence of DMSO.

**Figure 14** : Schematic representation of the plasmid Jx10AS-CMV-EF-pGL3.

**Figure 15** : Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with : (i) 10 ng of plasmid Jx10AS-CMV-pGL3 (**a**), or Jx10AS-CMV-EF-pGL3 (**b**), (ii) increasing quantities of plasmid pSG5-hPPARG2g2, and (iii) 20 ng of plasmid pRL-null. The activity of each inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

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**Figure 16** : Schematic representation of the plasmid Jx5AS-TK-luc-hPPARG2.

**Figure 17** : Schematic representation of the plasmid SV-g2-J10-C-pGL3.

**Figure 18** : Schematic representation of the plasmid hPPARG2-CMV-Jx5AS-TK-pGL3.

**Figure 19** : Schematic representation of the plasmid hPPARG2-CMV-Jx10AS-CMV-pGL3.

**Figure 20** : Comparison of the different versions of the system inducible in vitro. Mouse myoblasts (C2C12) are transfected with, for each version of the system, the same number of moles of inducible expression cassettes. The results are expressed as a percentage of the activity of the hCMV-IE promoter obtained using the plasmid pCMV-leadTK. The factors for induction with BRL49653 are calculated by dividing the activity in the presence of BRL49653 with the activity in the presence of DMSO. 1 = pSG5-hPPARG2 + Jx5AS-TK-pGL3; 2 = Jx5AS-TK-luc-hPPARG2; 3 = pSG5-hPPARG2g2 + Jx10AS-CMV-pGL3; 4 = pSG5-hPPARG2 + Jx10AS-CMV-pGL3; 5 = SV-g2-J10-C-pGL3; 6 = hPPARG2-CMV-Jx5AS-TK-pGL3; 7 = hPPARG2-CMV-Jx10AS-CMV-pGL3; 8 = hPPARG2-CMV-Jx15AS-CMV-pGL3; 9 = hPPARG2-CMV-Jx20AS-CMV-pGL3.

**Figure 21** : Comparison in vitro of the ligands BRL49653 and RG12525. Mouse myoblasts (C2C12) are transfected with: (i) 10 ng of plasmid hPPARG2-CMV-Jx10AS-CMV-pGL3  
15 whose expression cassette is presented in (a) and (ii) 10 ng of plasmid pRL-null. (b) The activity of the inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

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**Figure 22** : Comparison of the different versions of the system inducible in vivo. C57BI/6 mice (6 mice per group) are injected bilaterally, in their cranial tibial, with, for each version of the system, the same  
15 number of moles of inducible expression cassettes. An electrotransfer is then applied on each muscle. The treated animals receive each day, by force-feeding, 30 mg/kg of BRL49653. Four days after the injection of DNA, the animals are sacrificed and the muscles are  
20 removed in order to measure the luciferase activity.  
1 = pCMV-leadTK; 2 = pSG5-hPPARG2 + Jx10AS-CMV-pGL3;  
3 = pSG5-hPPARG2g2 + Jx10AS-CMV-pGL3; 4 = hPPARG2-CMV-Jx10AS-CMV-pGL3.

25 **Figure 23** : Comparison, in vivo, of various protocols for induction with BRL49653. C57BI/6 mice (6 mice per group) are injected bilaterally, in the cranial tibial,

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with 10 µg of DNA containing 1 µg of plasmid hPPARG2-CMV-Jx10AS-CMV-pGL3 whose expression cassette is presented in (a). The activities obtained with the various induction protocols are assembled in the panel

5 (b).

**Figure 24** : Schematic representation of the plasmid pRDA02.

10 **Figure 25** : Kinetics of induction obtained in vivo with the inducible system. (A) Ten C57BI/6 mice are injected bilaterally, in the cranial tibial, with a DNA mixture containing 3 mg of plasmid pRDA02 and 3 mg of plasmid pSG5-hPPARG2. An electrotransfer is then applied on

15 each muscle. Four days, and then 39 days after the injection of DNA, the animals are treated, by force-feeding, with 30 mg/kg of BRL49653. At various times, blood samples are collected over heparin and the enzymatic activity of the secreted alkaline phosphatase

20 (hSeAP) is measured in the plasma, using the Phospha-Light™ kit (Tropix, PE Biosystems, Foster City, CA). (B) C57BI/6 mice (2 groups of 10 mice) are injected bilaterally, in the cranial tibial, with a DNA mixture containing 3 mg of plasmid pRDA02 and 3 mg of plasmid

25 pSG5-hPPARG2. An electrotransfer is then applied on each muscle. Four days after the injection of DNA, the animals receive, by force-feeding, either a single dose

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of BRL49653 (30 mg/kg), or one dose per day (30 mg/kg) for 5 days. At various times, blood samples are collected over heparin, and the enzymatic activity of hSeAP is measured in the plasma, using the "Phospha-  
5 Light" kit (Tropix). The results presented (induction factors) correspond to the ratio between the hSeAP activity measured on the day of interest and that obtained on D4.

- 10 **Figure 26:** Comparison, *in vivo*, of different PPAR $\gamma$  ligands, and study of the dose effect of one of them. C57BI/6 mice (5 mice per group) are injected bilaterally, in the cranial tibial, with a DNA mixture containing 5 mg of plasmid pRDA02 and 5 mg of plasmid  
15 pSG5-hPPAR $\gamma$ 2. An electrotransfer is then applied on each muscle. Six days (**A**) or 10 days (**B**) after the injection of DNA, the animals are treated, by force-feeding, either with different PPAR $\gamma$  ligands (**A**; BRL49653, Actos<sup>TM</sup> (Takeda Pharmaceuticals) and Avandia<sup>TM</sup>  
20 (SmithKline Beecham)), or with various doses of BRL49653 (**B**). At various times, blood samples are collected over heparin, and the enzymatic activity of hSeAP is measured in the plasma, using the "Phospha-  
Light" kit (Tropix). The results presented (induction  
25 factors) correspond to the ratio between the hSeAP activity measured on the day of interest and that obtained on D6 (**A**) or D10 (**B**).

**Figure 27:** Schematic representation of the plasmid Jx10AS-CMV-VEGF<sub>A</sub>165.

## MATERIALS AND METHODS

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The methods conventionally used in molecular biology, such as preparative extractions of plasmid DNA, caesium chloride gradient centrifugation of plasmid DNA, electrophoresis on agarose gels, purification of DNA fragments by electroelution, precipitation of plasmid DNA in saline medium with ethanol or isopropanol, transformation in Escherichia coli are well known to persons skilled in the art and are abundantly described in the literature (Sambrook et al., "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989).

The plasmid pGL3-Basic, used for the clonings of the various promoter regions, as well as the plasmid PRL-null, are of commercial origin (Promega Corporation). The plasmids pSG5 (Stratagene), pBluescript II SK+ (Stratagene) and pSL301 (Invitrogen Corporation) are also of commercial origin. The constructions of the expression plasmids pSG5-hPPARG2 (Fajas L. et al., *J. Biol. Chem.*, **272** (1997) 18779-18789) and pSG5-hPPARA(Koz) (Cervois P. et al., *Mol.*

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*Endocrinol.*, **13** (1999) 400-409) have previously been described.

The construction of the plasmid pCMV-leadTK has also previously been described in patent application FR 98/120000 of 25/09/98, and in patent application US SN 60/123,298 (provisional application).

It is recalled that this plasmid is constructed in the following manner. The expression vector pCGN previously described by Tanaka et al. (Cell, **60** (1990) 375-386) contains the CMV promoter (-522/+72) fused with the "leader" of the HSV tk gene (+51/+101) upstream of a sequence encoding the haemagglutinin epitope. The plasmid pCGN (10 ng) was used as template for a PCR amplification. The primers which were used are the following:

- Primer 6718

(5'CCC GTTACATAACTTACG GTAAATGGCCCG3') (SEQ ID NO : 26), this primer hybridizes with the CMV promoter at position -522 (8 nucleotides downstream of the EcoRI site of pCGN).

- primer 6719

(5'GGGACGCGCTTCTACAAGCGCTGCGCCGAA3') (SEQ ID NO : 27), this primer hybridizes up to position 101 of the tk "leader". The first nucleotide G in bold is intended to restore the NcoI site of pGL3-Basic as will be explained below.



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The PCR fragment thus obtained is purified and then phosphorylated with the aid of the T4 phage polynucleotide kinase (New England Biolabs). In parallel, the vector pGL3-Basic (Promega) was linearized with NcoI, purified and then treated with Klenow DNA polymerase (Boehringer Mannheim) so as to fill the NcoI site. This vector is then dephosphorylated with the aid of alkaline phosphatase (Boehringer Mannheim) and then used for the insertion of the phosphorylated PCR fragment. Thus, the guanosine (G) of the primer 6719 makes it possible to restore only the NcoI site when the CMV-tk leader fragment is oriented with the 5' part (primer 6718, position -522 of the CMV) downstream of the HindIII site of pGL3-Basic and its 3' end (primer 6719, tk leader) is ligated to the NcoI site of pGL3-Basic (first ATG of luciferase). The plasmid thus obtained is designated pCMV-leadTK.

The enzymatic amplification of DNA fragments by the PCR (polymerase chain reaction) technique may be carried out using a DNA thermal cycler<sup>TM</sup> (Perkin Elmer Cetus) according to the manufacturer's recommendations.

The electroporation of plasmid DNA into Escherichia coli cells may be carried out with the aid of an electroporator (Bio-Rad) according to the manufacturer's recommendations.

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The verification of the nucleotide sequences may be carried out by the method developed by Sanger et al., (*Proc. Natl. Acad. Sci. USA*, **74** (1977) 5463-5467) using the kit distributed by Applied Biosystems according to the manufacturer's recommendations.

The murine myoblasts C2C12 are cultured in DMEM™ medium (Life Technologies Inc.) supplemented with 10% foetal calf serum (FCS). The cultures are carried out in an oven at 37°C, under a humid atmosphere and at a CO<sub>2</sub> partial pressure of 5%.

The transfections are carried out in 24-well plates and each transfection is carried out three times. Twenty-four hours after the transfection, the cells are inoculated at  $3 \times 10^4$  cells per well in DMEM™ medium. For each well, 500 ng of plasmid DNA (plasmids of interest and pBluescript II SK+ in order to adjust to 500 ng) are mixed with the cationic lipid RPR120535 B (WO 97/18185) in an amount of 6 nmol of lipid per µg of DNA in DMEM™ medium (20 µl final) comprising 150 mM NaCl and 50 mM bicarbonate. After 20 minutes at room temperature, the 20 µl of the DNA/lipid mixture are brought into contact with the cells, in the absence of FCS, for 2 hours. The culture medium is then supplemented with FCS or with ULTROSER™ (BioSeptra Inc.) so as to obtain a final concentration of 10% or 2% respectively. The PPAR ligands, dissolved in DMSO, are added to the culture medium at the same time as the FCS

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or the ULTROSER™. Forty-eight hours after the transfection, the culture medium is removed and the cells are rinsed twice with PBS (Life Technologies Inc.). The activity of the Photinus pyralis luciferase and the activity of the Renilla reniformis luciferase are then determined with the aid of the Dual-Luciferase Reporter Assay System™ kit (Promega Corporation) according to the supplier's recommendations.

The in vivo gene transfer experiments are carried out on 6-week-old C57BI/6 female mice. The animals are anaesthetized with 250 µl of a ketamine (Rhône Mérieux, 10 mg/ml final)/Xylazine (Bayer Pharma, 0.3 mg/ml final) mixture by the intraperitoneal route. An injection of a total quantity of 10 µg of DNA is then carried out into each cranial tibial muscle. Each leg is then subjected to an electric field (frequency of 1 Hz; 4 pulses of 20 ms at 250 V/cm). During the entire duration of the experiment, the animals receive each morning, by force-feeding, either 30 mg/kg of BRL49653 (SmithKline Beecham) in 1% carboxycellulose (weight/volume), or 1% carboxycellulose alone. Four days after the gene transfer, the animals are sacrificed and the muscles collected in PLB™ lysis buffer (Promega Corporation) in Lysing Matrix™ tubes (BIO 101, Inc.). The grinding of the muscles, which makes it possible to extract luciferase, is carried out with the aid of the FastPrep™ apparatus (BIO 101, Inc.)

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for 25 seconds at 6.5 m/s. The activity of the Photinus pyralis luciferase is then determined with the aid of the Luciferase Assay System™ kit (Promega Corporation) according to the supplier's recommendations.

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### EXAMPLES

**EXAMPLE 1 : Construction of promoters inducible by the PPARs and of expression plasmids containing them.**

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#### 1.1. Plasmid FTKpGL3.

A DNA fragment, corresponding to part of the promoter of the TK gene of the type 1 herpes simplex virus (HSV-1), between positions -105 and +56 relative to the site of initiation of transcription, was amplified by PCR using the plasmid pBLCAT2 (Luckow B. and Schutz G., *Nucleic Acids Res.*, **15** (1987) 5490) as template and the oligonucleotides 5' CGA CTC TAG AAG ATC TTG CCC CGC CCA GCG 3' (SEQ ID NO:28) and 5' TCG CCA AGC TTC TCG TGA TCT GCG GCA 3' (SEQ ID NO:2) as primers. This fragment was digested with BglII and HindIII and was then cloned into the plasmid pGL3-Basic previously digested with BglII and HindIII in order to obtain the plasmid FTKpGL3. A schematic representation of this plasmid is presented in Figure 1.

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## 1.2 Plasmids JxnS-TK-pGL3.

A DNA fragment, containing one or more (n) J sites of the promoter of the human ApoA-II gene, was amplified by PCR using the plasmid J3TKpGL3 (Vu-Dac N. et al., *J. Clin. Invest.*, **96** (1995) 741-750) as template and the oligonucleotides 3RDA37 (5' ACG TGT CGA CAC TAG TGG CTA GAG GAT CTC TAC CAG G 3'; SEQ ID NO:3) and 4RDA48 (5' CGA TGG TAC CCT CGA GCA ATG TGC TAG CGA GAT CCT TCA ACC TTT ACC 3'; SEQ ID NO:4) as primers. This fragment was digested with XhoI and SpeI and was then cloned into the plasmid FTKpGL3 previously digested with XhoI and NheI, in the direction of transcription of the minimal TK promoter (S), in order to obtain the plasmids Jx1S-TK-pGL3, Jx2S-TK-pGL3 and Jx3S-TK-pGL3, depending on the number of J sites present. A schematic representation of the plasmid Jx3S-TK-pGL3 is presented in Figure 2.

The DNA fragment, amplified by PCR using the plasmid J3TKpGL3 and the oligonucleotides 3RDA37 and 4RDA48 as primers, digested with XhoI and SpeI, was also cloned into the plasmid Jx3S-TK-pGL3 previously digested with XhoI and NheI, in order to obtain the plasmids Jx4S-TK-pGL3, Jx5S-TK-pGL3 and Jx6S-TK-pGL3, depending on the number of J sites present.

### 1.3. Plasmids JxnAS-TK-pGL3.

The plasmids JxnAS-TK-pGL3 differ from the plasmids JxnS-TK-pGL3 by the orientation of the J sites present in the inducible promotor. A DNA fragment containing one or more J sites of the promoter of the human ApoA-II gene, was amplified by PCR using the plasmid J3TKpGL3 as template and the oligonucleotides 3RDA37 and 4RDA48 as primers. This fragment was digested with SalI and NheI and was then cloned, in the opposite direction of transcription of the minimal TK promotor (AS), into the plasmid FTKpGL3 previously digested with XhoI and NheI in order to obtain the plasmids Jx1AS-TK-pGL3, Jx2AS-TK-pGL3 and Jx3AS-TK-pGL3, depending on the number of J sites present. A schematic representation of the plasmid Jx3AS-TK-pGL3 is presented in Figure 3.

The DNA fragment, amplified by PCR using the plasmid J3TKpGL3 and the oligonucleotides 3RDA37 and 4RDA48 as primers, digested with KpnI and SpeI, was also cloned in the antisense (AS) orientation into the plasmid Jx3AS-TK-pGL3 previously digested with KpnI and NheI in order to obtain the plasmids Jx4AS-TK-pGL3 and Jx5AS-TK-pGL3 depending on the number of J sites present.

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#### 1.4 Plasmids DR1xnS-TK-pGL3.

These plasmids contain, as PPAR response element (PPRE), a consensus sequence (AGGTCA A AGGTCA, SEQ ID NO:5) called consensus DR1. A DNA fragment, containing one or more consensus DR1 sites, was amplified by PCR using the oligonucleotides 1RDA69 (5' ACG TGT CGA CAC TAG TCA AAA CTA GGT CAA AGG TCA CGG AAA ACT AGG TCA AAG GTC ACG GAG AAC TAG 3'; SEQ ID NO:6) and 2RDA64 (5' CGA TGG TAC CCT CGA GCA ATG TGC TAG CCG TGA CCT TTG ACC TAG TTT TCC GTG ACC TTT GAC C 3'; SEQ ID NO:7) as primers. This fragment was digested with XhoI and SpeI and was then cloned, in the sense orientation, into the plasmid FTKpGL3 previously digested with XhoI and NheI in order to obtain the plasmids DR1x2S-TK-pGL3 and DR1x3S-TK-pGL3, depending on the number of consensus DR1 sites present. A schematic representation of the plasmid DR1x3S-TK-pGL3 is presented in Figure 4.

The DNA fragment, amplified by PCR using the oligonucleotides 1RDA69 and 2RDA64 as primers, digested with XhoI and SpeI, was also cloned into the plasmid DR1x3S-TK-pGL3 previously digested with XhoI and NheI in order to obtain the plasmids DR1x5S-TK-pGL3, DR1x6S-TK-pGL3 and DR1x7S-TK-pGL3, depending on the number of consensus DR1 sites present.

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### 1.5. Plasmids DR1xnAS-TK-pGL3.

The plasmids DR1xnAS-TK-pGL3 differ from the plasmids DR1xnS-TK-pGL3 by the orientation of the consensus DR1 sites present in the inducible promoter. A DNA fragment, containing one or more consensus DR1 sequences, was amplified by PCR using the oligonucleotides 1RDA69 and 2RDA64 as primers. This fragment was digested with SalI and NheI, and was then cloned, in the antisense orientation, into the plasmid FTKpGL3 previously digested with XhoI and NheI in order to obtain the plasmids DR1x2AS-TK-pGL3 and DR1x3AS-TK-pGL3, depending on the number of consensus DR1 sites present. A schematic representation of the plasmid DR1x3AS-TK-pGL3 is presented in Figure 5.

The DNA fragment, amplified by PCR using oligonucleotides 1RDA69 and 2RDA64 as primers, digested with KpnI and SpeI, was also cloned into the plasmid DR1x3AS-TK-pGL3 previously digested with KpnI and NheI in order to obtain the plasmids DR1x5AS-TK-pGL3 and DR1x6AS-TK-pGL3 depending on the number of consensus DR1 sites present.

### EXAMPLE 2 : Specificity of the PPARs for different response elements.

#### 2.1. System using hPPAR $\gamma$ 2.



The activity of the inducible promoters, using hPPARG2 as transcriptional regulator, was evaluated in transient transfection in mouse myoblasts (Figure 6). The results show that, depending on the response element (PPRE) used, the induction by the hPPARG2 ligand (BRL49653) and the final activity after activation vary. The best results were obtained using J sites as PPRE. Furthermore, the orientation of the PPRE is also important. In the case of the J site, the AS orientation is more favourable (Panel c).

## 2.2. System using hPPARa.

The results obtained with hPPARa as transcriptional regulator are assembled in Figure 7. Unlike hPPARG2, it is the consensus DR1 which is the best PPRE for hPPARa (Panels d and e).

These results therefore show (1) the functionality of the plasmids of the invention and (2) that depending on the PPAR chosen in the inducible system, it is important to select the PPRE most appropriate for the transcriptional regulator. This choice may influence the induction factor due to the presence of the ligand but also the level of activity reached after induction. It is understood that other PPREs can be used in the system of the invention.

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**EXAMPLE 3: Construction of promoters inducible by the PPARs containing a minimum promoter other than that of HSV1-TK such as for example the hCMV-IE minimum promoter.**

**3.1. Construction of the plasmids containing the hCMV-IE minimum promoter.**

A DNA fragment, containing the hCMV-IE minimum promoter (from position -54 to position +48 relative to the site of initiation of transcription), was amplified by PCR using the plasmid pCMV $\beta$  (Clontech) as template and the oligonucleotides 5RDA32 (5' ACG TAG ATC TCG GTA GGC GTG TAC GGT GGG AG 3'; SEQ ID NO:8) and 6RDA29 (5' ACG TAA GCT TCT ATG GAG GTC AAA ACA GC 3'; SEQ ID NO:9) as primers. This fragment was digested with HindIII and BglII and was then cloned into the plasmid FTKpGL3 previously digested with HindIII and BglII in order to obtain the plasmid FCMVpGL3.

The plasmid Jx5AS-TK-pGL3 was digested with BglII and NheI in order to isolate the BglII-NheI fragment of 179 bp containing 5 copies of the J site. This fragment was inserted into the plasmid FCMVpGL3 previously digested with BglII and NheI in order to give the plasmid Jx5AS-CMV-pGL3. A schematic

representation of the plasmid Jx5AS-CMV-pGL3 is presented in Figure 8.

The plasmid Jx5AS-CMV-pGL3 was digested with SphI and NheI in order to isolate the SphI-NheI fragment of 982 bp containing 5 copies of the J site, the hCMV-IE minimum promoter and the 5' part of the gene encoding luciferase. This fragment was inserted into the plasmid Jx5AS-CMV-pGL3 previously digested with SphI and SpeI in order to give the plasmid Jx10AS-CMV-pGL3. The plasmids Jx15AS-CMV-pGL3 and Jx20AS-CMV-pGL3 were also obtained by following the same strategy.

**3.2. Activity of plasmids containing the hCMV-IE minimum promoter.**

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A comparison of the minimum promoters which can be used in the inducible system was made in transient transfection. The results, which are assembled in Figure 9, show that depending on the minimum promoter, the final activity after induction may vary by a factor of 2. These results show in particular that, under the conditions tested, the CMV promoter appears to give a higher activity. Of course other minimum promoters, such as promoters not containing a TATA box, may be used.

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**EXAMPLE 4 : Importance of the number of response elements present in the inducible promoters.**

The optimization of the number of PPRES present in the inducible promoter was studied in transient transfection. The results, presented in Figure 10, show that the higher the number of copies of the PPRE, the greater the induction factor for the ligand and the activity induced. On the other hand, if this number is too high, both the induction factor and the induced activity decrease, this being regardless of the quantity of hPPARG2 present in the assay (Figure 11). The optimum number of PPRE appears to be between 10 and 15.

15

**EXAMPLE 5: Construction of a transcriptional regulator highly inducible by the PPAR ligands.**

**5.1. Construction of a transcriptional**  
regulator comprising two copies of the ligand-binding domain. Construction of the plasmid pSG5-hPPARG2g2.

A DNA fragment, noted A, containing the DNA region complementary to hPPARG2 encoding the C-terminal part of the F domain, was amplified by PCR using the plasmid pSG5-hPPARG2 as template and the oligonucleotides 20RDA21 (5' GGT TTG CTG AAT GTG AAG CCC 3'; SEQ ID NO:10) and 21RDA42 (5' AGT CTC TAG AGC

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TAC GCG TAC AAG TCC TTG TAG ATC TCC TGC 3'; SEQ ID NO:11) as primers. A DNA fragment, noted B, containing the DNA region complementary to hPPARg2 encoding the E and F domains, was amplified by PCR using the plasmid pSG5-hPPARg2 as template and the oligonucleotides 22RDA32 (5' AGT CAC GCG TGG GCG ATC TTG ACA GGA AAG AC 3'; SEQ ID NO:12) and 23RDA21 (5' GCC TTT GAG TGA GCT GAT ACC 3'; SEQ ID NO:13) as primers. The A fragment, digested with SacI and MluI and the B fragment, digested with MluI and XbaI, were cloned together into the plasmid pSG5-hPPARg2 previously digested with SacI and XbaI in order to obtain the plasmid pSG5-hPPARg2g2. This plasmid, whose schematic representation is presented in Figure 12, contains a complementary DNA which encodes a transcriptional regulator (noted hPPARg2g2) comprising two copies of the E and F domains, that is to say two ligand-binding domains.

The complete sequence of PPARy2y2 is represented below (SEQ ID NO:24):

MGETLGDSPI DPESDSFTDTLSANISQEMTMVDMPTMPFWPTNFGISSVDLSVMEDHSHSFDI  
 KPFTTVDFSSISTPHYEDI PFTRTDPVVADYKYDLKQEQSAIKVEPASPPYYSEKTQLYN  
 KPHEEPSNSLMAIECRVCGDKASGFHYGVHACEGCKGFFRRTIRLKLIIYDRCDLNCRIHKKS  
 RNKCQYCRFQKCLAVGMSHNAIRFGRMPPQAEKEKLLAEISSDIDQLNPESADLRALAKHYLD  
 SYIKSPPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIF  
 QGCQFRSVEAQVEITEYAKSIPGFVNLDLNDQVTLKYGVEIIYTMASLNMKNDGVLISEG  
 QGFMTRFLKSLRKPFQDFMEPKFEFAVKFNALDSDLAIFIAVILSGDRPGLLNKPI  
 EDIQDNLQALELQLKLNHPSSQLFAKLLQKMTDLRQIVTEHVQLLQVIKKTETDMSLHPL  
 LQEIYKDLAYAWAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIFQGC  
 QFRSVEAQVEITEYAKSIPGFVNLDLNDQVTLKYGVEIIYTMASLNMKNDGVLISEGGF  
 MTRFLKSLRKPFQDFMEPKFEFAVKFNALDSDLAIFIAVILSGDRPGLLNKPIEDI  
 QDNLQALELQLKLNHPSSQLFAKLLQKMTDLRQIVTEHVQLLQVIKKTETDMSLHPLLEQ  
 IYKDLV

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The sequence of the C-terminal part of PPAR $\gamma$ 2,  
comprising the E and F domains, is the following  
sequence SEQ ID NO:25:

```
MMGEDKIKFKHITPLQEQSKEVAIRIFQGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTL
LKYGVHEIIYTMLASLMNKDGVLI SEGQGFMTREFLKSRLKPPGDFMEPKFEFAVKFNALEL
DDSDLAIFI AVIILSGDRPGLLNVPIDIQDNLQALELQKL NHPESQLFAKLLQKMTD
LRQIVTEHVQLLQVIKKTETDMSLHPLLQEITYKDLYAWAILTGKTTDKSPFVIYDMSLMMG
EDKIKFKHITPLQEQSKEVAIRIFQGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLKY
GVHEIIYTMLASLMNKDGVLI SEGQGFMTREFLKSRLKPPGDFMEPKFEFAVKFNALELDDS
DLAIFI AVIILSGDRPGLLNVPIDIQDNLQALELQKL NHPESQLFAKLLQKMTDLRQ
IVTEHVQLLQVIKKTETDMSLHPLLQEITYKDLY
```

5

## 5.2. Activity of the plasmid pSG5-hPPAR $\gamma$ 2g2.

The results presented in Figure 13 show that  
if the induced activity is lower using hPPAR $\gamma$ 2g2 as  
transcriptional regulator (Figure 13 **a** and **b**), the  
induction factor for the ligand (Figure 13 **c**) is much  
higher with this regulator. The difference between the  
two transcriptional regulators is explained by the fact  
that for hPPAR $\gamma$ 2g2, the background noise of the system  
in the absence of ligand is low and remains low,  
regardless of the quantity of regulator present. On the  
other hand, the higher the increase in hPPAR $\gamma$ 2g2, the  
higher the induced activity, which is not the case for  
the system using hPPAR $\gamma$ 2 which appears to saturate.

The presence of a second ligand-binding  
domain (hPPAR $\gamma$ 2g2) therefore confers on the  
transcriptional regulator greater inducibility by the  
ligand.

**EXAMPLE 6 : Increase in the final activity of the inducible promoters.**

6.1. Construction of an inducible expression  
5 cassette comprising the intron of hEF1a. Construction  
of the plasmid Jx10AS-CMV-EF-pGL3.

A DNA fragment, containing the first intron  
of the gene encoding hEF1a (from position +16 to  
10 position +984 relative to the site of initiation of  
transcription; Genbank accession number: E02627), was  
amplified by PCR using the oligonucleotides 25RDA35 (5'  
AGT CAC TAG TAA GCT TTT TGC CGC CAG AAC ACA GG 3'; SEQ  
ID NO:14) and 26RDA36 (5' AGT CAC TAG TCC ATG GCT GCC  
15 CAG TGC CTC ACG ACC 3'; SEQ ID NO:15) as primers. This  
fragment was digested with HindIII and NcoI and was  
then cloned into the plasmid Jx10AS-CMV-pGL3 previously  
digested with HindIII and NcoI in order to obtain the  
plasmid Jx10AS-CMV-EF-pGL3. A schematic representation  
20 of the plasmid Jx10AS-CMV-EF-pGL3 is presented in  
Figure 14.

6.2. Activity of the plasmid Jx10AS-CMV-EF-  
pGL3.  
25

With the aim of increasing the final activity  
of the system, an enhancer sequence, situated in the

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first intron of the hEFla gene, was cloned in the vicinity of the inducible promoter. The results presented in Figure 15 show that the presence of the enhancer region increases the induced activity of the system, this being regardless of the quantity of transcriptional regulator used.

**EXAMPLE 7 : Construction of plasmids comprising both a cassette for expression of the transcriptional regulator and an inducible expression cassette.**

#### 7.1. Plasmid Jx5AS-TK-luc-hPPARg2.

The plasmid pSG5-hPPARa(Koz) was digested with MluI and ScaI in order to isolate the MluI-ScaI fragment of 1229 bp containing the 3' region of the DNA complementary to hPPARa. This fragment was inserted into the plasmid pSL301 previously digested with MluI and SmaI in order to give the plasmid pSL-3'hPPARa.

The plasmid pSG5-hPPARa(Koz) was digested with SalI and MluI in order to isolate the SalI-MluI fragment of 1406 bp containing the SV40 virus early promoter and the 5' region of the DNA complementary to hPPARa. This fragment was inserted into the plasmid pSL-3'hPPARa previously digested with XhoI and MluI in order to give the plasmid pSL-hPPARa.



The plasmid pSL-hPPARA was digested with SpeI and SalI in order to isolate the SpeI-SalI fragment of 2664 bp containing the SV40 virus early promoter and the DNA complementary to hPPARA. This fragment was  
5 inserted into the plasmid pBluescript II SK+ previously digested with SpeI and SalI in order to give the plasmid pBS-hPPARA.

The plasmid pSG5-hPPARG2 was digested with AvrII and SacI in order to isolate the AvrII-SacI  
10 fragment of 2070 bp, noted C, containing the 5' region of the DNA complementary to hPPARG2. A DNA fragment, noted D, containing the 3' region of the DNA complementary to hPPARG2, was amplified by PCR using the plasmid pSG5-hPPARG2 as template and the  
15 oligonucleotides 10RDA21 (5' CAG GTT TGC TGA ATG TGA AGC 3'; SEQ ID NO:16) and 11RDA40 (5' TGA CGT GTC GAC CTA GTA CAA GTC CTT GTA GAT CTC CTG C 3'; SEQ ID NO:17) as primers. The C fragment and the D fragment, digested with SacI and SalI, were cloned together into the  
20 plasmid pBS-hPPARA previously digested with AvrII and SalI in order to obtain the plasmid pBS-hPPARG2.

The plasmid Jx5AS-TK-pGL3 was digested with KpnI and SalI in order to isolate the KpnI-SalI fragment of 2324 bp containing the luc+ gene under the  
25 control of an inducible promoter. This fragment was inserted into the plasmid pBS-hPPARG2 previously digested with KpnI and SalI in order to give the

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plasmid Jx5AS-TK-luc-hPPARG2. A schematic representation of the plasmid Jx5AS-TK-luc-hPPARG2 is presented in Figure 16.

5                    **7.2. Plasmid SV-g2-J10-C-pGL3.**

The plasmid pBS-hPPARG2 was digested with NotI and SalI in order to isolate the NotI-SalI fragment of 2622 bp, noted E, containing the DNA  
10 complementary to hPPARG2 under the control of the SV40 early promoter. A DNA fragment, noted F, containing the SV40 virus polyadenylation site, was amplified by PCR using the plasmid FTK-pGL3 as template and the oligonucleotides 18RDA31 (5' AGT CGT CGA CGC TTC GAG  
15 CAG ACA TGA TAA G 3'; SEQ ID NO:18) and 19RDA35 (5' AGT CGC TAG CGA CGG ATC CTT ATC GAT TTT ACC AC 3'; SEQ ID NO:19) as primers. The E fragment and the F fragment, digested with SalI and NheI, were cloned together into the plasmid Jx10AS-CMV-pGL3 previously digested with  
20 NotI and NheI in order to obtain the plasmid SV-g2-J10-C-pGL3. A schematic representation of the plasmid SV-g2-J10-C-pGL3 is presented in Figure 17.

25                    **7.3. Plasmid hPPARG2-CMV-Jx5AS-TK-pGL3.**

A DNA fragment, noted G, containing the DNA complementary to hPPARG2, was amplified by PCR using

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the plasmid Jx5AS-TK-luc-hPPArg2 as template and the oligonucleotides 12RDA50 (5' GTC AGC TAG CCT ACT CGA GCC ACC ATG GGT GAA ACT CTG GGA GAT TCT CC 3'; SEQ ID NO:20) and 13RDA42 (5' TAC GGG GTA CCC AGA CAT GAT AAG ATA CAT TGA TGA GTT TGG 3'; SEQ ID NO:21) as primers. A DNA fragment, noted H, containing the hCMV-IE minimum promoter (from position -54 to position +48 relative to the site of initiation of transcription), was amplified by PCR using the plasmid pCMV $\beta$  as template and the oligonucleotides 14RDA33 (5' GTC AGC TAG CCG GTA GGC GTG TAC GGT GGG AGG 3'; SEQ ID NO:22) and 15RDA33 (5' TAC GCT CGA GCT TCT ATG GAG GTC AAA ACA GCG 3'; SEQ ID NO:23) as primers. The G fragment, digested with KpnI and XhoI and the H fragment, digested with XhoI and NheI, were cloned together into the plasmid Jx5AS-TK-pGL3 previously digested with KpnI and NheI in order to obtain the plasmid hPPArg2-CMV-Jx5AS-TK-pGL3. A schematic representation of the plasmid hPPArg2-CMV-Jx5AS-TK-pGL3 is presented in Figure 18.

20

#### 7.4. Plasmids hPPArg2-CMV-Jx5AS-CMV-pGL3.

The plasmid Jx5AS-CMV-pGL3 was digested with NheI and SphI in order to isolate the NheI-SphI fragment of 982 bp containing the 5' region of the luc+ gene under the control of an inducible promoter. This fragment was inserted into the plasmid hPPArg2-CMV-

Jx5AS-TK-pGL3 previously digested with SpeI and SphI in order to give the plasmid hPPARG2-CMV-Jx10AS-CMV-pGL3. A schematic representation of the plasmid hPPARG2-CMV-Jx10AS-CMV-pGL3 is presented in Figure 19.

- 5           The plasmid Jx5AS-CMV-pGL3 was digested with NheI and SphI in order to isolate the NheI-SphI fragment of 982 bp containing the 5' region of the luc+ gene under the control of an inducible promoter. This fragment was inserted into the plasmid hPPARG2-CMV-Jx10AS-CMV-pGL3 previously digested with SpeI and SphI in order to give the plasmid hPPARG2-CMV-Jx15AS-CMV-pGL3.

- The plasmid Jx10AS-CMV-pGL3 was digested with NheI and SphI in order to isolate the NheI-SphI fragment of 1151 bp containing the 5' region of the luc+ gene under the control of an inducible promoter. This fragment was inserted into the plasmid hPPARG2-CMV-Jx10AS-CMV-pGL3 previously digested with SpeI and SphI in order to give the plasmid hPPARG2-CMV-Jx20AS-CMV-pGL3.

**EXAMPLE 8 : Comparison of the different versions of the inducible system in vitro.**

- 25           Figure 20 assembles the results obtained in vitro with various versions of the inducible system. These results show that the systems using two plasmids

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(Figure 20, lines 1, 3 and 4) like the systems with only one plasmid (Figure 20, lines 2 and 5 to 9) are functional; that is to say that the presence of a PPARG ligand (here BRL49653) greatly increases the expression of the gene placed under the control of the inducible promoter. It is also observed that for some systems (Figure 20, lines 3 and 7 to 9), the induction factor for the ligand is greater than 30, and that for the system presented in Figure 20, line 4, the activity after induction is equal to that of a strong promoter such as that of the hCMV-IE promoter.

**EXAMPLE 9 : Various PPAR ligands can activate the inducible system**

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**9.1. System using hPPARG2.**

The results presented in Figure 21 show that hPPARG ligands other than BRL49653, here RG12525 (RPR ligand for hPPARG), may be used to activate the inducible system. At a concentration of 100  $\mu$ m, a treatment with RG12525 even leads to a higher induction than that obtained with BRL49653. Any other PPARG ligand may therefore be used as inducer of the system.

25

## 9.2. System using hPPAR $\alpha$ .

In the same manner as for the system using hPPAR $\gamma$ , a system using hPPAR $\alpha$  as transcriptional  
5 regulator may be activated with the fibrates or WY-14,643 for example or any other hPPAR $\alpha$  ligand.

**EXAMPLE 10 : The inducible system may be activated in vivo, in the muscle.**

10

Figure 22 assembles the results obtained in vivo, in the muscle, with different versions of the inducible system. The results show that for the three versions tested (Figure 22, lines 2 to 4), a treatment  
15 by force-feeding with a hPPAR $\gamma$  ligand is capable of greatly increasing, in the muscle, the activity of the inducible promoters. The induction factors are: x14 for the Figure 22 line 2 version, x8 for the Figure 22 line 3 version, and x24 for the Figure 22 line 4 version.  
20 Furthermore, for one of the versions (Figure 22, line 2), the activity obtained in the animals treated with BRL49653 is of the order of that of a strong promoter such as the hCMV-IE promoter.

The results, presented in Figure 23, also  
25 show that a single dose of ligand can induce the system, whether this dose is taken before or after the gene transfer. This experiment also shows that a dose

which is two times smaller than that normally used makes it possible to obtain the same induction factor.

The system, using a PPAR nuclear receptor as transcriptional regulator, is therefore functional in vivo and may be induced by the oral administration of a PPAR ligand.

**EXAMPLE 11: Construction of a plasmid allowing the inducible expression of a gene whose product is secreted.**

#### 11.1 Construction of the plasmid pRDA02

The plasmid Jx10AS-CMV-pGL3 was digested with HindIII and MluI in order to isolate the HindIII-MluI fragment of 459 bp. This fragment was inserted into the plasmid pXL3010 (Bettan M. et al., *Anal. Biochem.*, **271** (1999) 187-189) previously digested with HindIII and MluI in order to give the plasmic pRDA02. This plasmid contains the DNA complementary to the gene encoding the secreted form of human placental alkaline phosphatase (hSeAP) whose expression is under the control of a promoter inducible by the system using the PPARs as transcriptional regulator. A schematic representation of the plasmid pRDA02 is presented in Figure 24.

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**EXAMPLE 12: The inducible system makes it possible to regulate, in vivo, the plasma concentration of a secreted protein.**

5           The results presented in Figure 25 show that, by using the inducible system, it is possible to regulate, over time, the plasma concentration of a protein secreted from the muscle, this being with a simple oral administration of a PPAR ligand. The plasma  
10 concentration of hSeAP is increased by a factor of 18 (Figure 25A) two days after the administration of ligand, and then returns to its base level one week later. Between the 21st and 39th day, an immune response directed against hSeAP of human origin is  
15 observed and results in a decrease in the plasma concentration of this protein. Despite this immune response, it is possible to carry out a second induction cycle (Figure 25A).

          As shown in Figure 25B, the inducible system  
20 also makes it possible, by daily administrations of ligand, to maintain the plasma level of hSeAP at a high level for a period equal to the duration of the treatment.



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**EXAMPLE 13:** Various PPAR ligands can activate the inducible system in vivo, this being in a dose-dependent manner.

5           BRL49653, in its commercial form for the treatment of type II diabetes (Avandia™, SmithKline Beecham) and pioglitazone, in its commercial form for this same treatment (Actos™, Takeda Pharmaceuticals) can also activate the inducible system (Figure 26A).  
10 Figure 26B also shows that the induction factor is directly correlated with the dose of ligand used.

          The system, using a PPAR nuclear receptor as transcriptional regulator, therefore makes it possible to control, very precisely, the plasma level of a  
15 secreted protein. Furthermore, this regulation may be obtained using various PPAR ligands.

**EXAMPLE 14:** Construction of a plasmid allowing the inducible expression of a gene whose product is an  
20 angiogenic factor.

          14.1 Construction of the plasmid Jx10AS-CMV-VEGF<sub>A</sub>165.

25           The human VEGF165 reading frame was cloned by reverse transcription and PCR from total RNA of human placenta (Clontech) (Houck et al. *Mol. Endocrinol.* 12

(1991) 1806-1814) and then inserted into a plasmid pBluescript (Stratagene) containing the CMV E/P promoter from position -522 to +72 and the SV40 late polyA, in order to give the plasmid pXL3218. The latter  
5 was then digested with HindIII and BsrGI in order to isolate the HindIII-BsrGI fragment A of 482 bp. The plasmid pXL3218 was also digested with BsrGI and BamHI in order to isolate the BsrGI-BamHI fragment B of 390 bp. Fragments A and B were inserted into the  
10 plasmid Jx10AS-CMV-pGL3 previously digested with HindIII and BamHI in order to give the plasmid Jx10AS-CMV-VEGF<sub>A</sub>165. This plasmid contains the DNA complementary to the gene encoding VEGF<sub>A</sub>165 whose expression is under the control of a promoter inducible  
15 by the system using PPARs as transcriptional regulator. A schematic representation of the plasmid Jx10AS-CMV-VEGF<sub>A</sub>165 is presented in Figure 27.

This plasmid can be used, for example, to control, over time, the angiogenic activity of VEGF for  
20 therapeutic purposes.

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531 Rec'd PCT.

18 DEC 2001

## AMENDED CLAIMS

[received by the international office on 3 January 2001 (03.01.01);

original claims 1-30 replaced by new claims 1-33 (4 pages)]

- 5                   1.    Composition comprising:
- (a) a first element comprising a nucleic acid  
of interest under the control of an inducible promoter  
comprising a PPAR response element and a minimal  
transcriptional promoter, and
- 10                   (b) a second element comprising a nucleic  
acid encoding a PPAR under the control of a  
transcriptional promoter,  
for their use simultaneously, separately or spaced out  
over time.
- 15                   2.    Composition according to Claim 1,  
characterized in that it comprises in addition:
- (c) a ligand for PPAR,  
for a use simultaneously, separately or spaced out over  
time.
- 20                   3.    Composition according to Claim 1 or 2,  
characterized in that the elements (a) and (b) are  
carried by distinct genetic constructs.
4.    Composition according to Claim 1 or 2,  
characterized in that the elements (a) and (b) are
- 25 assembled in the same genetic construct.

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5. Composition according to Claim 3 or 4, characterized in that the genetic construct is a plasmid or viral vector.

6. Composition according to Claim 5, characterized in that the viral vector is an adeno-associated virus (AAV).

7. Composition according to one of Claims 1 to 6, characterized in that the PPAR response element comprises one or more PPAR-binding sites.

8. Composition according to Claim 7, characterized in that the PPAR response element comprises one or more sites having the sequence SEQ ID NO:1 or functional variants of this sequence.

9. Composition according to Claim 7, characterized in that the PPAR response element comprises one or more sites having the sequence SEQ ID NO:5 or functional variants of this sequence.

10. Composition according to Claims 7 to 9, characterized in that the response element comprises up to 30 binding sites, preferably from 3 to 20, more preferably from 5 to 15.

11. Composition according to one of Claims 1 to 10, characterized in that the minimal promoter is a promoter of a cellular or viral gene deleted for the region(s) not essential for transcriptional activity.

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12. Composition according to one of Claims 1 to 11, characterized in that the inducible promoter comprises, in addition, an enhancer region.

13. Composition according to one of Claims 1 to 12, characterized in that the minimal promoter and the PPAR response element are in the same orientation.

14. Composition according to one of Claims 1 to 12, characterized in that the minimal promoter and the PPAR response element are in the opposite orientation.

15. Composition according to one of Claims 1 to 14, characterized in that the nucleic acid encoding a PPAR encodes a PPAR $\alpha$  or a PPAR $\gamma$ .

16. Composition according to one of Claims 1 to 15, characterized in that the nucleic acid encoding a PPAR encodes a modified PPAR comprising several ligand-binding sites.

17. Composition according to one of Claims 1 to 16, characterized in that it comprises, in addition, an element (d) comprising a nucleic acid encoding an RXR under the control of a transcriptional promoter.

18. Vector comprising an element (a) and an element (b) according to Claim 1.

19. Vector according to Claim 18, characterized in that the elements (a) and (b) are in the opposite orientation.

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20. Vector according to Claim 18 or 19,  
characterized in that the inducible promoter of the  
element (a) and the transcriptional promoter of the  
element (b) are assembled in the vector to form a  
5 regulable bidirectional promoter.

21. Vector according to Claim 20,  
characterized in that it comprises, in the 5'→3'  
direction, a first nucleic acid encoding a PPAR, a  
first minimal transcriptional promoter controlling the  
10 expression of the said first nucleic acid, one or more  
PPAR response elements, a second minimal  
transcriptional promoter and, under the control of the  
said second minimal transcriptional promoter, a second  
nucleic acid encoding a product of interest.

15 22. Vector according to one of Claims 18 to  
21, characterized in that it comprises, in addition, an  
element (d) according to Claim 17.

23. Use of a composition according to one of  
Claims 1 to 17 or of a vector according to one of  
20 Claims 18 to 22 for expressing a nucleic acid of  
interest in a cell ex vivo or in vitro.

24. Use of a composition according to one of  
Claims 1 to 17 or of a vector according to one of  
Claims 18 to 22 for the preparation of a product  
25 intended for expressing a nucleic acid of interest in a  
cell in vivo.

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25. Method for the regulated expression of a nucleic acid in a cell, in vitro or ex vivo comprising bringing the said cell into contact with a composition according to one of Claims 1 to 17 or a vector

5 according to one of Claims 18 to 22.

26. Method according to Claim 25, characterized in that it is a mammalian, preferably human cell.

27. Method according to Claim 26,  
10 characterized in that it is a muscle cell.

28. Method for regulating the expression of a nucleic acid in vivo comprising the administration of a composition according to one of Claims 1 to 17 or of a vector according to one of Claims 18 to 22.

15 29. Cell modified by bringing into contact with a composition according to one of Claims 1 to 17 or a vector according to one of Claims 18 to 22.

30. Modified PPAR comprising several ligand-binding sites.

20 31. Nucleic acid encoding a PPAR according to Claim 30.

32. Method for identifying PPAR ligands, comprising the bringing of a cell according to Claim 29 into contact with a test molecule and the detection of  
25 the expression of the nucleic acid of interest.

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33. Method for identifying PPAR ligands in vivo, characterized in that there is administered a composition according to one of Claims 1 to 17 or a vector according to one of Claims 18 to 22 as well as a  
5 test molecule, and in that the expression of the nucleic acid of interest is detected.



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ABSTRACT OF THE DISCLOSURE

Provided herein are novel methods and compositions for the pharmacological regulation of the expression of transgenes

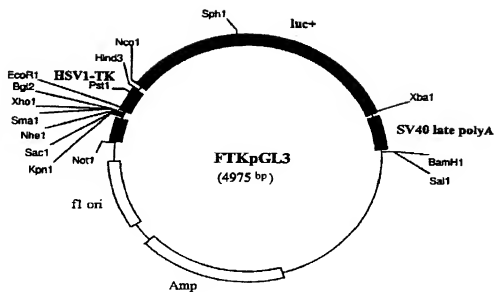


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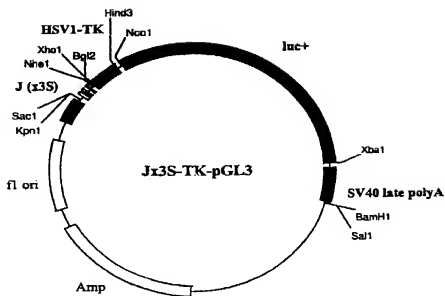


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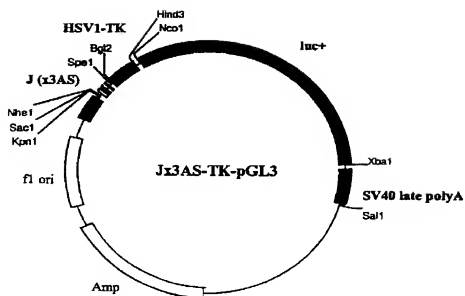


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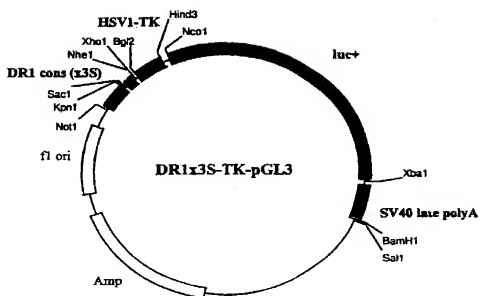


FIGURE 4

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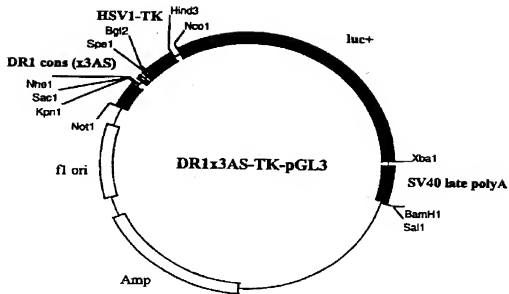


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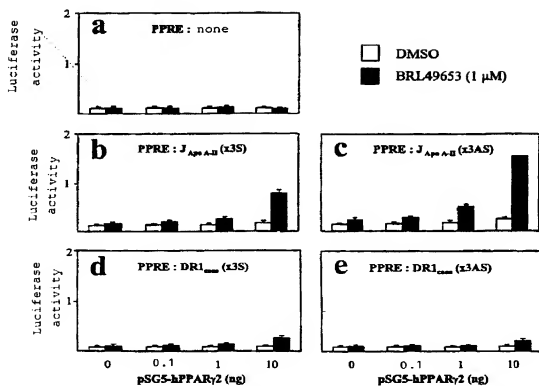


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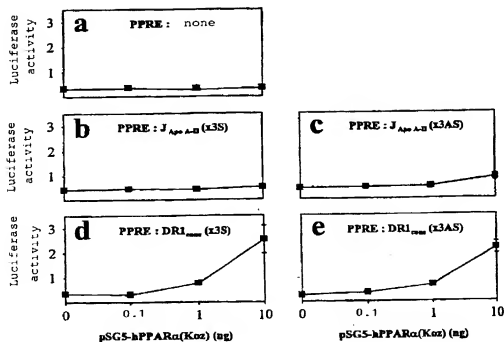


FIGURE 7



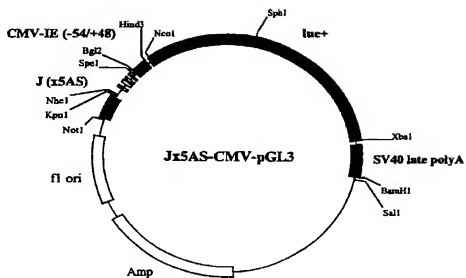


FIGURE 8

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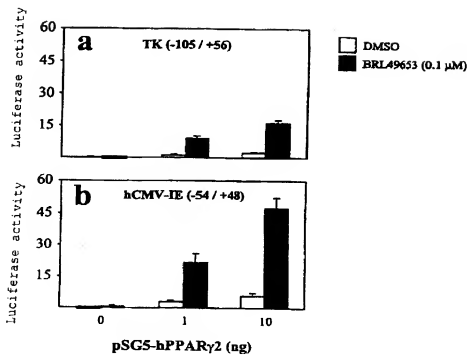


FIGURE 9

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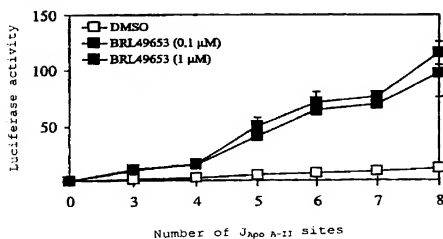


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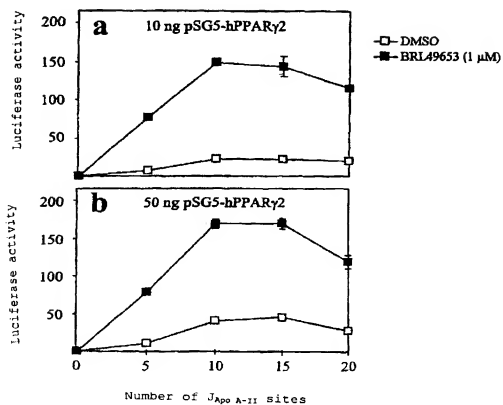


FIGURE 11

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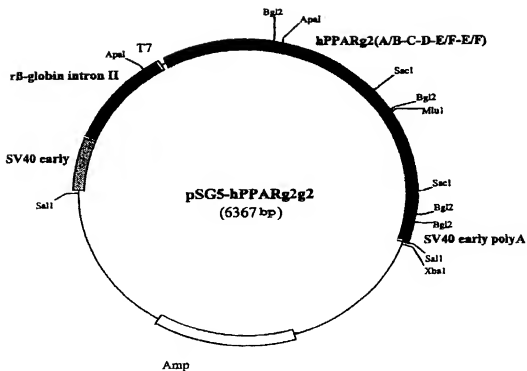


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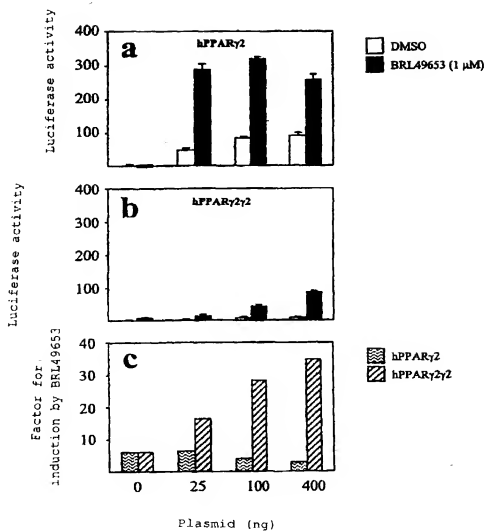


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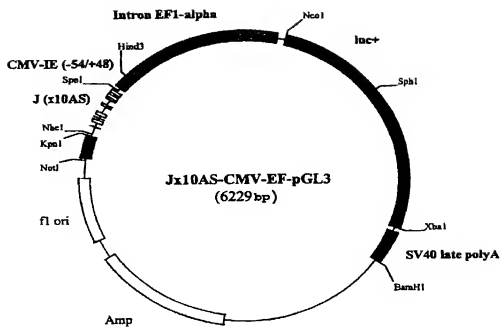


FIGURE 14

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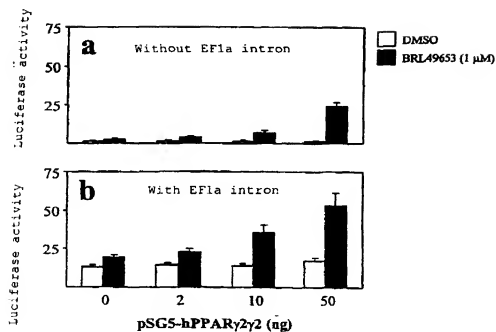


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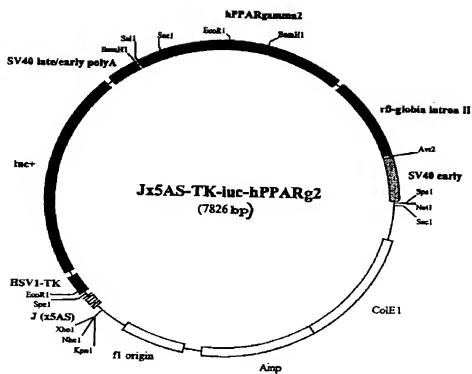


FIGURE 16

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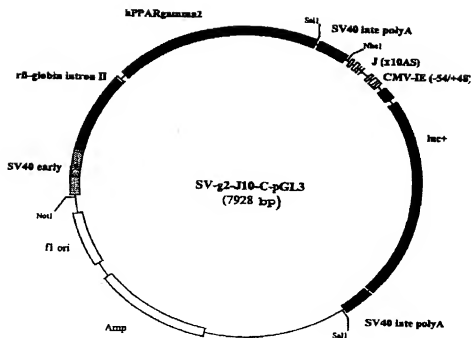


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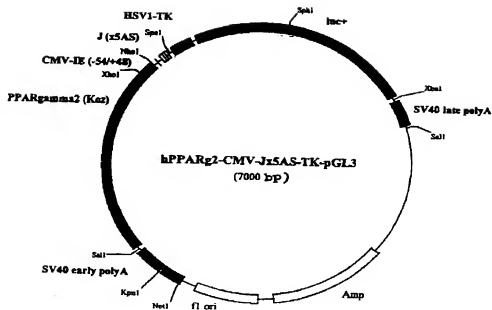


FIGURE 18

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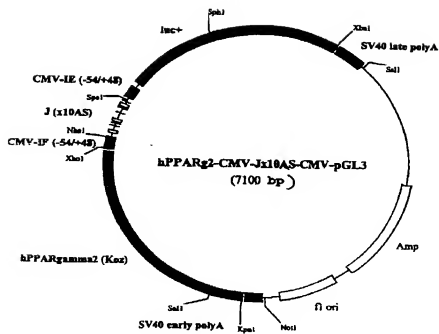


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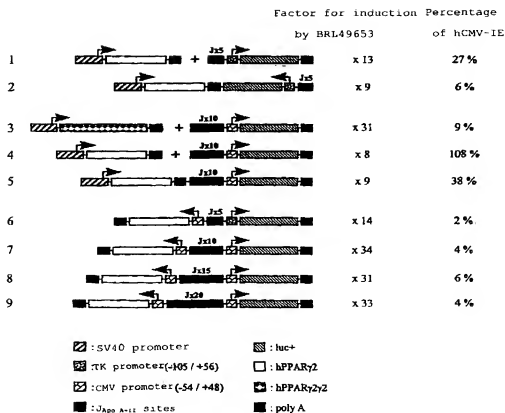


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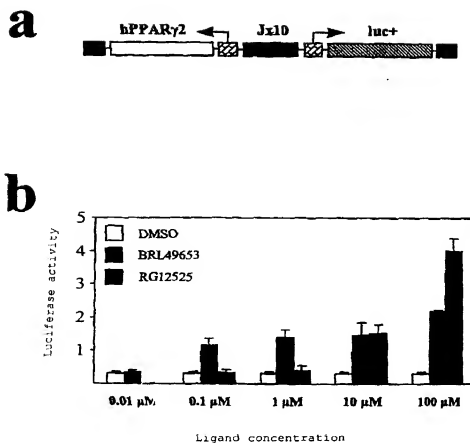


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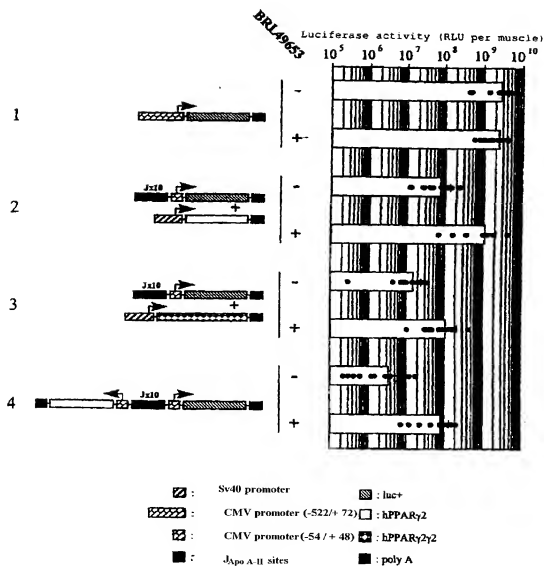


FIGURE 22

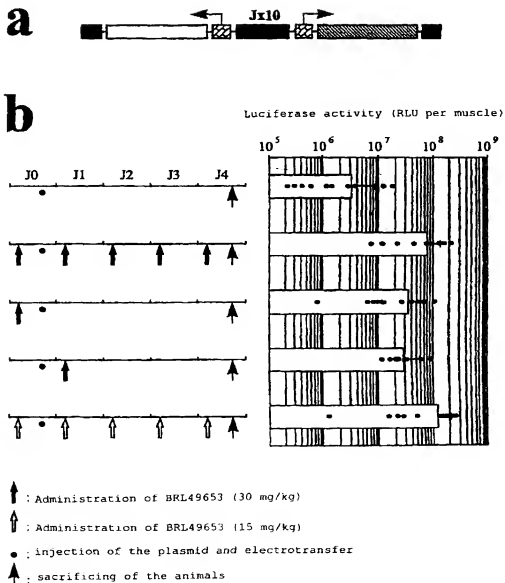


FIGURE 23



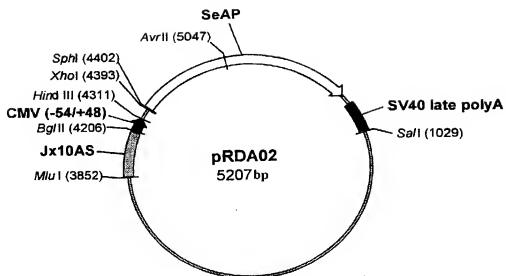


FIGURE 24

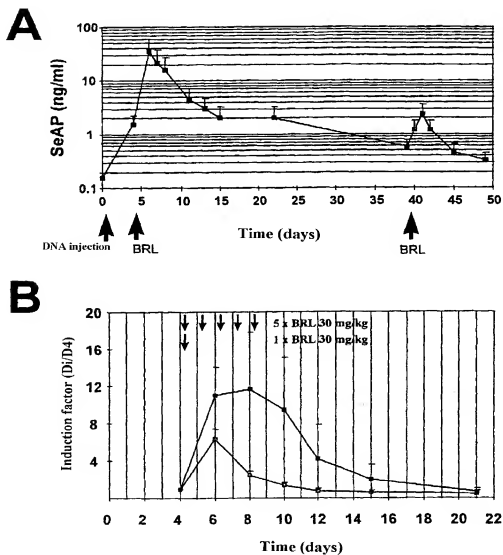


FIGURE 25

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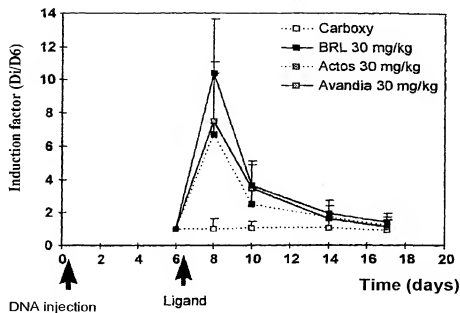
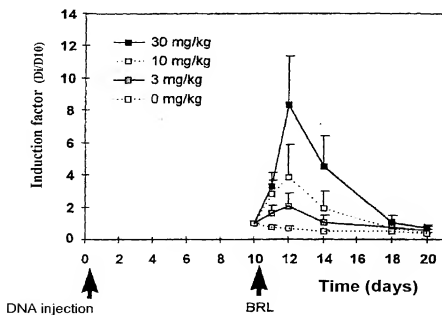
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FIGURE 26

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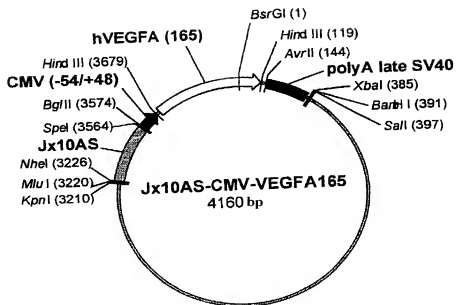


FIGURE 27

UNITED STATES OF AMERICA

# DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor(s), I/We hereby declare that:

My/Our residence(s), post office address(es) and citizenship(s) are as stated below my name(s).  
I/We verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

## SYSTEM FOR REGULATION OF EXPRESSION USING PPAR NUCLEAR RECEPTORS

and the specification of which  
(check one)

- ☐ Is attached hereto (Attorney Docket No. **ST99021 US PCT**)  
☒ was filed on **December 18, 2001** as U.S. Application Number **10/018,729**  
 and was amended on \_\_\_\_\_ (if applicable).  
☐ was described and claimed in PCT Int'l Application Number filed on \_\_\_\_\_  
 and as amended under PCT Article 19 on \_\_\_\_\_ (if any).

I/We hereby state that I/We have reviewed and understand the contents of the above identified specification, including the claims as amended by any amendment referred to above.  
 I/We acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 C.F.R. 1.56.



I/We hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or 365 (b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one other country other than the United States of America, listed below and having a filing date before that of the application on which priority is claimed. I/We have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Foreign  
Priority:

**99 07957**  
Number

**France**  
Country

**22 June 1999**  
Day/Month/Year Filed

Prior  
Foreign  
Appln(s):

Number

Country

Day/Month/Year Filed



I/We hereby claim the benefit under Title 35, United States Code §119(e) of any United States Provisional application(s) listed below:

**60/149,721**  
Number

**August 20, 1999**  
Filing Date



I/We hereby claim the benefit under Title 35, United States Code §120 or 365(c) of any United States application(s) or international application designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I/We acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

**PCT/FR00/01744**  
Application Serial No.

**June 22, 2000**  
Filing Date

**Completed**  
Status (Patented, Pending)

I/We hereby appoint the attorneys and/or agents associated with the Customer No.(s) provided below as my/our attorneys and/or agents with full power to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Customer No.: 005487

I/We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code §1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

inventor(s): ☐ Additional names and signatures are attached.

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Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Country of Citizenship: \_\_\_\_\_

Residence: \_\_\_\_\_  
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P. O. Address: \_\_\_\_\_

6. Full name: \_\_\_\_\_

Signature: \_\_\_\_\_

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P. O. Address: \_\_\_\_\_

7. Full name: \_\_\_\_\_

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8. Full name: \_\_\_\_\_

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

Country of Citizenship: \_\_\_\_\_

Residence: \_\_\_\_\_  
(City and State/City and Country only)

P. O. Address: \_\_\_\_\_

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Bridgewater, NJ 08807-0800  
Telephone (908) 231-4854  
Telefax (908) 231-2626

Docket ST99021 US PCT

10/018729  
531 Rec'd PCT 18 DEC 2001  
PCT/FR00/01744

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## SEQUENCE LISTING

&lt;110&gt; AVENTIS PHARMA S.A.

<120> System for the pharmacological regulation of expression  
using PPAR nuclear receptors and their ligands

## &lt;130&gt; SEQUENCES

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Arg Phe Gly Arg Met Pro Gln Ala Glu Lys Glu Lys Leu Leu Ala Glu  
 210 215 220  
 Ile Ser Ser Asp Ile Asp Gln Leu Asn Pro Glu Ser Ala Asp Leu Arg  
 225 230 235 240  
 Ala Leu Ala Lys His Leu Tyr Asp Ser Tyr Ile Lys Ser Phe Pro Leu  
 245 250 255  
 Thr Lys Ala Lys Ala Arg Ala Ile Leu Thr Gly Lys Thr Thr Asp Lys  
 260 265 270  
 Ser Pro Phe Val Ile Tyr Asp Met Asn Ser Leu Met Met Gly Glu Asp  
 275 280 285  
 Lys Ile Lys Phe Lys His Ile Thr Pro Leu Gln Glu Lys Lys Glu  
 290 295 300  
 Val Ala Ile Arg Ile Phe Gln Gly Cys Gln Phe Arg Ser Val Glu Ala  
 305 320 315 320  
 Val Gln Glu Ile Thr Glu Tyr Ala Lys Ser Ile Pro Gly Phe Val Asn  
 325 330 335  
 Leu Asp Leu Asn Asp Gln Val Thr Leu Leu Lys Tyr Gly Val His Glu  
 340 345 350  
 Ile Ile Tyr Thr Met Leu Ala Ser Leu Met Asn Lys Asp Gly Val Leu  
 355 360 365  
 Ile Ser Glu Gly Gln Gly Phe Met Thr Arg Glu Phe Leu Lys Ser Leu  
 370 375 380  
 Arg Lys Pro Phe Gly Asp Phe Met Glu Pro Lys Phe Glu Phe Ala Val  
 385 390 395 400  
 Lys Phe Asn Ala Leu Glu Leu Asp Asp Ser Asp Leu Ala Ile Phe Ile  
 405 410 415  
 Ala Val Ile Ile Leu Ser Gly Asp Arg Pro Gly Leu Leu Asn Val Lys  
 420 425 430  
 Pro Ile Glu Asp Ile Gln Asp Asn Leu Leu Gln Ala Leu Glu Leu Gln  
 435 440 445  
 Leu Lys Leu Asn His Pro Glu Ser Ser Gln Leu Phe Ala Lys Leu Leu  
 450 455 460  
 Gln Lys Met Thr Asp Leu Arg Gln Ile Val Thr Glu His Val Gln Leu  
 465 470 475 480  
 Leu Gln Val Ile Lys Lys Thr Glu Thr Asp Met Ser Leu His Pro Leu  
 485 490 495  
 Leu Gln Glu Ile Tyr Lys Asp Leu Tyr Ala Trp Ala Ile Leu Thr Gly

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500	505	510
Lys Thr Thr Asp Lys Ser Pro Phe Val Ile Tyr Asp Met Asn Ser Leu		
515	520	525
Met Met Gly Glu Asp Lys Ile Lys Phe Lys His Ile Thr Pro Leu Gln		
530	535	540
Glu Gln Ser Lys Glu Val Ala Ile Arg Ile Phe Gln Gly Cys Gln Phe		
545	550	555
Arg Ser Val Glu Ala Val Gln Glu Ile Thr Glu Tyr Ala Lys Ser Ile		
565	570	575
Pro Gly Phe Val Asn Leu Asp Leu Asn Asp Gln Val Thr Leu Leu Lys		
580	585	590
Tyr Gly Val His Glu Ile Ile Tyr Thr Met Leu Ala Ser Leu Met Asn		
595	600	605
Lys Asp Gly Val Leu Ile Ser Glu Gly Gln Gly Phe Met Thr Arg Glu		
610	615	620
Phe Leu Lys Ser Leu Arg Lys Pro Phe Gly Asp Phe Met Glu Pro Lys		
625	630	635
Phe Glu Phe Ala Val Lys Phe Asn Ala Leu Glu Leu Asp Asp Ser Asp		
645	650	655
Leu Ala Ile Phe Ile Ala Val Ile Ile Leu Ser Gly Asp Arg Pro Gly		
660	665	670
Leu Leu Asn Val Lys Pro Ile Glu Asp Ile Gln Asp Asn Leu Leu Gln		
675	680	685
Ala Leu Glu Leu Gln Leu Lys Leu Asn His Pro Glu Ser Ser Gln Leu		
690	695	700
Phe Ala Lys Leu Leu Gln Lys Met Thr Asp Leu Arg Gln Ile Val Thr		
705	710	715
Glu His Val Gln Leu Leu Gln Val Ile Lys Lys Thr Glu Thr Asp Met		
725	730	735
Ser Leu His Pro Leu Leu Gln Glu Ile Tyr Lys Asp Leu Tyr		
740	745	750

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 <211> 467  
 <212> PRT  
 <213> Homo sapiens  
 <400> 25

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Met Met Gly Glu Asp Lys Ile Lys Phe Lys His Ile Thr Pro Leu Gln  
 1 5 15

Glu Gln Ser Lys Glu Val Ala Ile Arg Ile Phe Gln Gly Cys Gln Phe  
 20 25 30

Arg Ser Val Glu Ala Val Gln Glu Ile Thr Glu Tyr Ala Lys Ser Ile  
 35 40 45

Pro Gly Phe Val Asn Leu Asp Leu Asn Asp Gln Val Thr Leu Leu Lys  
 50 55 60

Tyr Gly Val His Glu Ile Ile Tyr Thr Met Leu Ala Ser Leu Met Asn  
 65 70 75 80

Lys Asp Gly Val Leu Ile Ser Glu Gly Gln Gly Phe Met Thr Arg Glu  
 85 90 95

Phe Leu Lys Ser Leu Arg Lys Pro Phe Gly Asp Phe Met Glu Pro Lys  
 100 105 110

Phe Glu Phe Ala Val Lys Phe Asn Ala Leu Glu Leu Asp Ser Ser Asp  
 115 120 125

Leu Ala Ile Phe Ile Ala Val Ile Ile Leu Ser Gly Asp Arg Pro Gly  
 130 135 140

Leu Leu Asn Val Lys Pro Ile Glu Asp Ile Gln Asp Asn Leu Leu Gln  
 145 150 155 160

Ala Leu Glu Leu Gln Leu Lys Leu Asn His Pro Glu Ser Ser Gln Leu  
 165 170 175

Phe Ala Lys Leu Leu Gln Lys Met Thr Asp Leu Arg Gln Ile Val Thr  
 180 185 190

Glu His Val Gln Leu Leu Gln Val Ile Lys Lys Thr Glu Thr Asp Met  
 195 200 205

Ser Leu His Pro Leu Leu Gln Glu Ile Tyr Lys Asp Leu Tyr Ala Trp  
 210 215 220

Ala Ile Leu Thr Gly Lys Thr Thr Asp Lys Ser Pro Phe Val Ile Tyr  
 225 230 235 240

Asp Met Asn Ser Leu Met Met Gly Glu Asp Lys Ile Lys Phe Lys His  
 245 250 255

Ile Thr Pro Leu Gln Glu Gln Ser Lys Glu Val Ala Ile Arg Ile Phe  
 260 265 270

Gln Gly Cys Gln Phe Arg Ser Val Glu Ala Val Gln Glu Ile Thr Glu  
 275 280 285

Tyr Ala Lys Ser Ile Pro Gly Phe Val Asn Leu Asp Leu Asn Asp Gln

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9

290	295	300
Val Thr Leu Leu Lys Tyr Gly Val His Glu Ile Ile Tyr Thr Met Leu		
305	310	315 320
Ala Ser Leu Met Asn Lys Asp Gly Val Leu Ile Ser Glu Gly Gln Gly		
	325	330 335
Phe Met Thr Arg Glu Phe Leu Lys Ser Leu Arg Lys Pro Phe Gly Asp		
	340	345 350
Phe Met Glu Pro Lys Phe Glu Phe Ala Val Lys Phe Asn Ala Leu Glu		
	355	360 365
Leu Asp Asp Ser Asp Leu Ala Ile Phe Ile Ala Val Ile Ile Leu Ser		
	370	375 380
Gly Asp Arg Pro Gly Leu Leu Asn Val Lys Pro Ile Glu Asp Ile Gln		
	385	390 395 400
Asp Asn Leu Leu Gln Ala Leu Glu Leu Gln Leu Lys Leu Asn His Pro		
	405	410 415
Glu Ser Ser Gln Leu Phe Ala Lys Leu Leu Gln Lys Met Thr Asp Leu		
	420	425 430
Arg Gln Ile Val Thr Glu His Val Gln Leu Leu Gln Val Ile Lys Lys		
	435	440 445
Thr Glu Thr Asp Met Ser Leu His Pro Leu Leu Gln Glu Ile Tyr Lys		
	450	455 460
Asp Leu Tyr		
465		

<210> 26  
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 <212> DNA  
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<400> 26  
 cccgttccat aaattacggt aaatggcccg

30

<210> 27  
 <211> 30  
 <212> DNA  
 <213> Homo sapiens

<400> 27  
 gsgagcgct tctacaagge gctggccgaa

30

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<210> 29  
<211> 30  
<212> DNA  
<213> Homo sapiens

<400> 28  
cgactcttaga agatcttgcc ccgccccagcg

30